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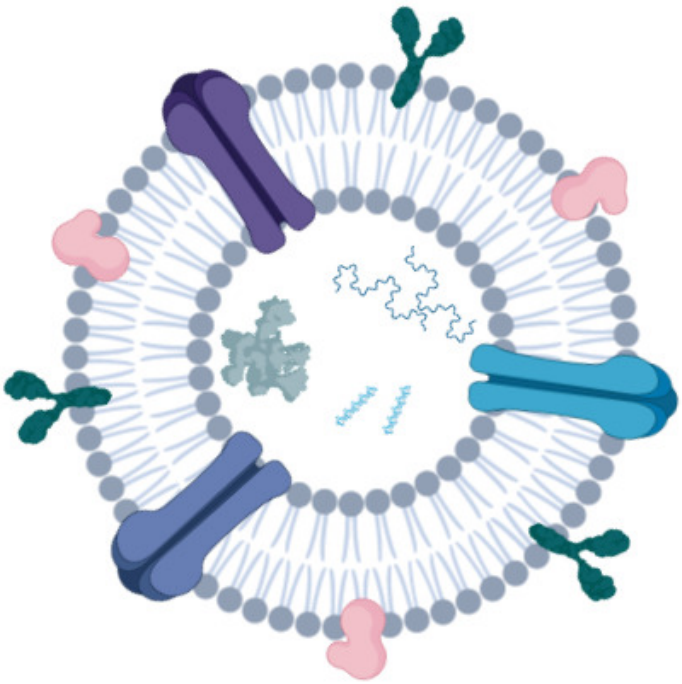
20-X2

Exosomes and lipid nanoparticles - the future of targeted drug delivery



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Abstract

In this project an overview of how synthetic lipid nanoparticles and exosomes can be used for targeted drug delivery is compiled. The goal is to identify aspects that can be in favor for targeted drug delivery and the development of products at Cytiva. The most important fields for Cytiva to understand is the methods and the challenges of cell culturing for production of exosomes, productions of lipid nanoparticles, purification of exosomes, analysis of both exosomes and lipid nanoparticles, and how exosomes and lipid nanoparticles are used as tools for drug delivery. To understand these aspects a description focusing on structural components, specific delivery and cargo loading is also included in the report. Many different components and methods have been found in the different fields mentioned, and the ones that we believe are the most relevant for Cytiva are presented and discussed in the report.

We conclude that both exosomes and lipid nanoparticle are suitable options as drug delivery vehicles, especially for their ability to be modified for targeted delivery, encapsulate therapeutic compounds and cross biological barriers. Exosomes are also biostable and possess low immunogenicity. For production the methods identified with highest potential are Hollow-Fiber Bioreactor for cell culturing in production of exosomes and Microemulsion and High-Pressure Homogenization for lipid nanoparticles. Purification is required for exosomes and the most prominent method is Size-Exclusion Chromatography, because of its scalability. After production and purification it is important to be able to detect the vesicles and the most developed and used methods are Nanoparticle Tracking Analysis and Flow Cytometry, beacuse they can use labeling techniques and single vesicle analysis.

Abbreviations

Abbrevation	Full name
ABC	Accelerated Blood Clearance
Alix	ALG-2 Interacting Protein X
APCs	Antigen-Presenting Cells
BBB	Blood-Brain Barrier
CD	Cluster of Differentiation
CMP	Cardiomyocyte Specific Peptides
DCs	Dendritic Cells
DDC	Drug Delivery Carries
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
ECS	Extracapillary Space
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMC	Extracellular Matrix
EPR	Enhanced Permeability and Retention Effect
EV	Extracellular Vesicle
EXPLOR	Exosomes for Protein Loading via Optically Reversible Protein-Protein Interactions
FA	Folate
Fab	Antigen-Binding Fragments
FBS	Fetal Bovine Serum
FCS	Fluorescence Correlation Spectroscopy
FDA	Federal Drug and Food Administration
FLOWER	Frequency Locking Optical Whispering Evanescent Resonator
FR	Folate Receptor
GM	Ganglioside-Monosialic Acid
GTPase	Guanosine Triphosphate Hydrolyse Enzymes
HCR	Hybridization Chain Reaction
HPH	High-Pressure Homogenization
HPLC	High Pressure Liquid Chromatography
Hsp	Heat Shock Protein
ICAM-1	Intercellular Adhesion Molecule-1
ICS	Intracapillary Space
Lamp	Lysosomal Associated Membrane Proteins
LNP	Lipid Nanoparticle

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Abbrevation	Full name
LCA	Life Cycle Assessment
lncRNA	Long Non-Coding RNA
LSPRi	Localized Surface Plasmon Resonance Imaging Platform
LTRS	Laser Tweezers Raman Spectroscopy
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
miRNA	MicroRNA
MPS	Mononuclear Phagocyte System
mRNA	Messenger RNA
MS	Mass Spectrometry
MSC	Mesenchymal Stem Cell
MVB	Multivesicular Bodies
MWCOs	Molecular Weight Cut-Offs
MWCNTs	Micropillar Arrays Functionalized with Multiwall Carbon Nanotubes
NC	Nanocarrier
NLC	Nanostructured Lipid Carriers
nPLEX	Nanoplasmonic Exosome Assay
NTA	Nanoparticle Tracking Analysis
PEG	Polyethylene Glycol
PCS	Photon Correlation spectroscopy
PDGFRs	Platelet-Derived Growth Factors
PIT	Phase Inversion Temperature
Rab	Ras-Related Proteins
RGD	Arginine-Glycine-Aspartic peptide
RNA	Ribonucleic Acid
RPS	Resistive Pulse Sensing
rRNA	Ribosomal RNA
RVG	Rabies Viral Glycoprotein
SEC	Size-Exclusion Chromatography
SERS	Surface Enhanced Raman Spectroscopy
SEM	Scanning Electron Microscopy
siRNA	Small Interfering RNA
SLN	Solid Lipid Nanoparticles
snRNA	Small Nuclear RNA
SPARTA	Single Particle Automated Raman Trapping Analysis

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Abbrevation	Full name
SP-IRIS	Single Particle Interferometric Reflectance Imaging Sensor
SPR	Surface Plasmon Resonance
ssDNA	Single-Stranded DNA
TAC	Tacrolimus Monohydrate
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TME	Tumor Microenviorment
tRNA	Transfer RNA
TRPS	Tuneable Resistive Pulse Sensing
TSG	Tumor Susceptibility Gene
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor Receptor
XPACK	Exosome Membrane-Anchored Protein

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1 Introduction

This project was commissioned by Cytiva, which is a company that develops and produce products and services for production and purification of biopharmaceutical products. To enable continued development and to be able to meet the demand on the market, Cytiva is interested in the field of exosomes and lipid nanoparticles as vehicles for targeted drug delivery. The most important fields for Cytiva to understand is the methods and the challenges of cell culturing for production of exosomes, productions of lipid nanoparticles, purification of exosomes and analysis of both exosomes and lipid nanoparticles. To gain the competence needed to enter this field, Cytiva also needs knowledge about structure of exosomes and lipid nanoparticles. In this report group 20-X2 will provide information regarding all these different parts together with challenges and opportunities in this field. The goal of this project is to compile a comprehensive analysis of exosomes and lipid nanoparticles as well as identify aspects that can be in favor for targeted drug delivery and the development of products at Cytiva.

2 Background

This section focus on exosome and lipid nanoparticle (LNP) structure and synthesis to give general information that is useful to understand the nature and function of these vesicles. Background regarding production and purification of exosomes can be found in Section 6.1 respectively 7 and production of LNP in Section 6.2. Regarding analysis and detection of exosomes and LNP, the background can be found in Section 8.1.

In recent years naturally occurring extracellular vesicles have been of interest as drug delivery systems due to their ability to communicate and deliver informational compounds to other cells (Piper & Katzmann 2007). Exosomes are nanospherical membrane structures formed by a bilayer of lipids as illustrated in Figure 1. Exosomes also contain different transmembrane components such as proteins, lipids and saccharides, as well as cytosolic proteins and nucleic acids (Vlassov *et al.* 2012). Currently there are more than 9700 proteins, 1100 lipids and 2600 nucleic acids known to be associated with exosomes, according to the exosome database ExoCarta (2020).

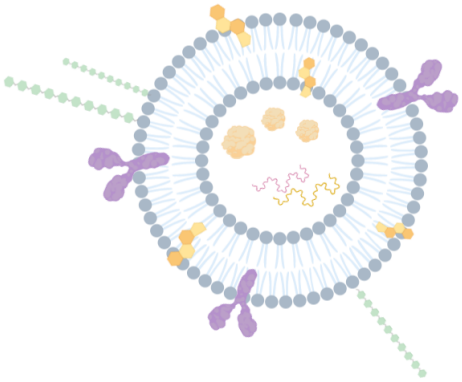

Figure 1. Simple illustration of an exosome. Exosomes are formed by a bilayer of lipids, and also contain components such as transmembrane proteins (in purple), saccharides (in green), cytosolic proteins in (yellow) and nucleic acids in (yellow and pink). Exosomes range in a size from 30-200 nm (van der Pol et al. 2014, Vlassov et al. 2012).

The biogenesis pathway of an exosome begins with late endosomes, so called multivesicular bodies, containing multiple internal vesicles that merge with the cell membrane and release its internal vesicles into the extracellular matrix (Piper & Katzmann 2007), see Figure 2. This biogenesis of exosomes via multivesicular bodies separates the exosomes from other extracellular vesicles such as apoptotic bodies and microvesicles (Théry *et al.* 2001).

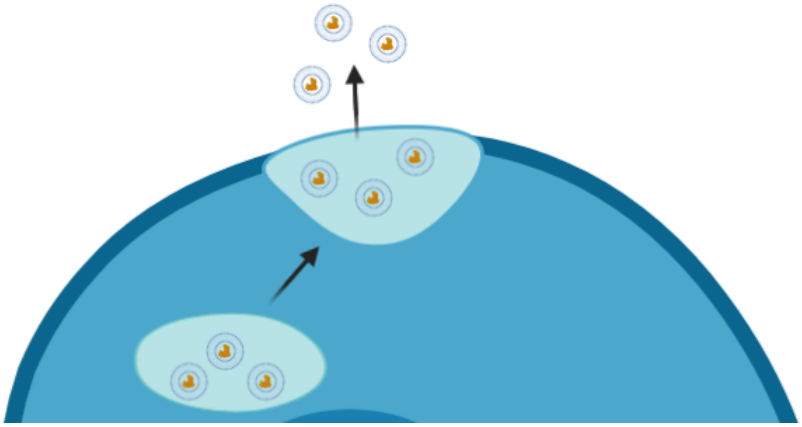

Figure 2. Illustration of exosomes and their biogenesis.

Synthetic nanoparticles have been made to mimic exosomes to overcome problems such as low drug loading capacity, large scale and time-consuming production obstacles as well as unfavorable economic exchange (Vázquez-Ríos *et al.* 2019). The solid lipid nanoparticle (SLN) is made out of solid crystalline lipid core embraced by a phospholipid coating. The hydrophobic core can be made out of different material including triglycerides, waxes and glyceride mixtures (Puri *et al.* 2009). The nanostructured lipid carrier (NLC) has a core matrix consisting of both liquid and solid lipids making the inner matrix more imperfect. The NLC is looked upon as a more developed SLN and both will be addressed in this report. The SLN and NLC possess the advantage of being able to encapsulate and deliver both hydrophobic and lipophobic cargo (Ghasemiyeh & Mohammadi-Samani 2018).

Exosomes range in size from 30-200 nm (van der Pol *et al.* 2014, Vlassov *et al.* 2012) and for the synthetic nanoparticles to also enjoy the privilege of enhanced permeability and retention (EPR) for facilitated accumulation and targeting, they are also made in the same size spectra (Kohli *et al.* 2014). Naturally, exosomes have the benefit of being able to deliver its cargo to specific cells due to its varying surface features which can be recognized by specific surface proteins (Zhang *et al.* 2019). In an attempt to emulate this biological interaction system LNPs can be decorated with surface-attached ligands which in turn is recognized by the targeted cells (Kohli *et al.* 2014, Yoo *et al.* 2019). Ligands including peptides, vitamins, affibody molecules and antibodies are commonly used in surface modifications for improved drug delivery (Yoo *et al.* 2019). Some of these modifications have also been performed on exosomes to improve their drug delivery potential (Johnsen *et al.* 2014, Xitong & Xiaorong 2016).

It is important to mention that way exosomes interact with their target cells is not fully understood. They have been shown to fuse with the membrane of target cells and thus releasing its contents into it (Edgar 2016). They have also been shown to be brought into cells using endocytosis. In some cases, they attach themselves to a cells surface to trigger a signal response inside the cell (Edgar 2016). These interactions are very important to understand if we want to use exosomes as a drug delivery vehicle. LNP are also engulfed by targeting cells and the most common drug release trigger is a change of pH, which is associated with inflammatory areas, tumor microenvironments (TME) and cancer cells (Chan *et al.* 2010, Chen *et al.* 2015).

Both exosomes and LNP are able carry a wide array of cargo protected inside a membrane (Zhang *et al.* 2019, Noble *et al.* 2014). This makes them suitable to transport materiel which would be susceptible to degradation in the extracellular matrix. These include proteins, lipids, mRNA, rRNA, miRNA, lncRNA (long non-coding RNA), and a wide array of other molecules (Zhang *et al.* 2019, Noble *et al.* 2014, Yi Xue *et al.* 2015, Martins *et al.* 2007). In exosomes these intracellular deliveries are thought to play a role in everything from intercellular communication to activating physiological responses (Zhang *et al.* 2019).

Despite these unknowns about the exosome it has gained a lot of attention as a potential way to deliver drugs to specific locations in the body. The synthetic made particles aiming to imitate exosomes are not without complications. Transmembrane and on-surface modifications needs to be done to increase the stability of LNP but the downside is the increased macrophage activity this generates (Mui *et al.* 2013, Zhang *et al.* 2016). The induced immunorespons is not necessarily toxic but it accelerate blood clearance and lower the concentration of the drug carrier to a level of no effectiveness (Zhang *et al.* 2016, Jokerst *et al.* 2011, Dai *et al.* 2018).

With further research, development and improvements of exosomes and LNP, both are future potential drug delivery particles for therapeutic treatments.

3 Exosomes as drug delivery vehicles

In this section elements for successful exosome-based drug delivery will be discussed, focusing on how target specificity can be enabled and enhanced making use of biological processes and biochemical exosomal composition and how specific cargo is loaded. The vesicular structure requires a certain stability in circulation, ensuring that the vehicle can be administered *in vivo* without degrading before reaching its specific target (Johnsen *et al.* 2014). The vesicle should be non-immunogenic and non-toxic to the host cell, and requires ability to carry a substantial amount of cargo to the recipient cells (Johnsen *et al.* 2014). Ability to specifically deliver drugs to recipient cells, enabling effective treatment without major side effects, requires targeting peptides on the surface of the vesicular unit (Johnsen *et al.* 2014). The method used to load the vesicle should not interfere or change the cargo or vesicle itself, since this can lower the efficiency of treatments (Johnsen *et al.* 2014).

Since exosomes are secreted naturally by many cell types, these vesicles are non-immunogenic and non-toxic to most host cells. The vesicle also have high biostability when distributed *in vivo*, and possess the ability to cross biological barriers such as the blood-brain barrier (BBB) (Tian *et al.* 2018b). Drug delivery vehicles that are able to cross the BBB enables drug delivery directly to the brain, potentially improving the success-rate of central nervous system drugs previously unsuccessful in clinical trials (Pardridge 2012). This report will address structure, production, purification and detection of exosomes and lipid nanoparticles.

3.1 Exosome targeted delivery

The structure and nature of exosomes enables them to encapsulate and deliver therapeutic cargo to recipient cells (Xitong & Xiaorong 2016). To be able to use exosomes as drug delivery vehicles one has to understand the mechanisms of this content delivery and how the delivery can be targeted towards specific recipient cells. These topics will be disclosed in the following section, with focus on modifications that enchances target specificity during cargo delivery.

The mechanisms of content delivery vary between different types of exosomes and recipient cells, but the most common mechanism for targeted drug delivery is membrane fusion via a ligand-receptor interaction (Kibria *et al.* 2018) as illustrated in Figure 3. When exosomes are administered *in vivo*, they will circulate through the host and accumulate at specific recipient cells bearing receptors matching with ligands on the exterior of the vesicle. This cell-cell communication between receptor and ligand will result in a fusion of exosome and recipient cell, and thus a release of exosomal cargo to the recipient cells (Xitong & Xiaorong 2016).

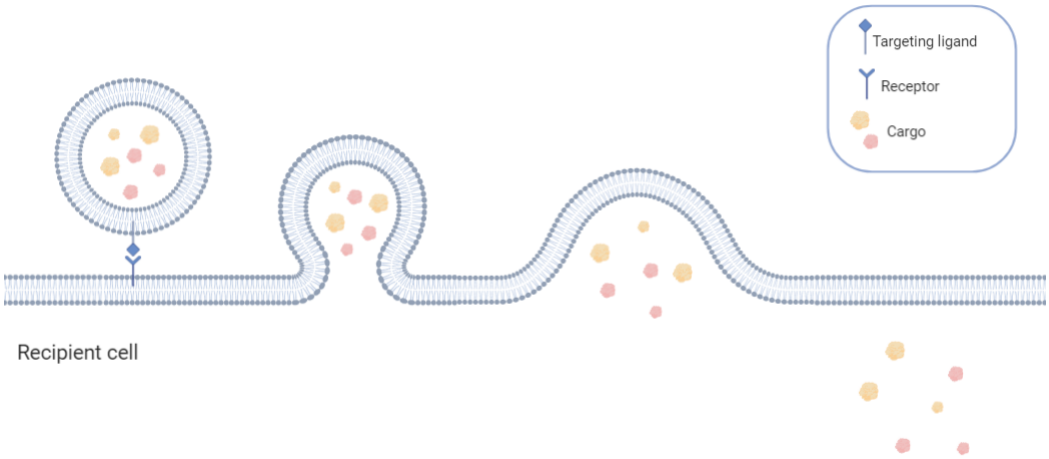

Figure 3. Membrane fusion via a ligand-receptor interaction. Interaction of exosome targeting ligands with receptors on recipient cells initiates a membrane-fusion event, resulting in release of exosome cargo into recipient cell.

3.1.1 Unmodified exosomes does not exhibit sufficient specificity

A challenge with using exosomes as vehicles for targeted drug delivery is being able to specifically deliver therapeutics to recipient cells whilst minimizing delivery and accumulation to other organs or tissues (Mentkowski & Lang 2019). Previous studies has shown that unmodified exosomes administered *in vivo* accumulates in liver, kidney and spleen before they are degraded during natural processes in the host cells (Smyth *et al.* 2015, Kooijmans *et al.* 2012). This results in minimal accumulation of pharmaceuticals in the targeted tissue as well as unwanted accumulation in other tissues and organs (Xitong & Xiaorong 2016). To increase target specificity and thus improve therapeutic efficiency, exosomes used as drug delivery vehicles can be bioengineered (Kibria *et al.* 2018).

3.1.2 Modifications of exosomes for increased target specificity

Exosome bioengineering is development of targeted exosomes for therapy, with the goal to increase target specificity by utilizing targeting molecules on the surface of the exosomes (Kibria *et al.* 2018). Target specificity can either be increased by inducing an overexpression of naturally occurring surface proteins, or by fusion of a targeting ligand with transmembrane proteins enriched in the exosomes (Johnsen *et al.* 2014, Xitong & Xiaorong 2016). By increasing specificity between targeting surface molecules and recipient cells, therapeutic efficiency can be improved and side effects minimized.

3.1.2.1 Enrichment of surface molecule by inducing overexpression in exosome donor cells

Since many exosome surface proteins reflects on both the origin and the target cell selection of exosomes, naturally occurring components can be utilized in directing exosomes toward specific tissues (Rana *et al.* 2012). By inducing an overexpression of a particular protein in the exosome donor cell, the same protein will be enriched on the exosomal surface and thus increase target specificity (Johnsen *et al.* 2014).

3.1.2.2 Display of targeting ligands by fusion to exosomal membrane protein

An increase of target specificity can be achieved by fusing a customized targeting peptide with a transmembrane protein that is naturally enriched in the exosome (Kibria *et al.* 2018). The fusion results in a display of the targeting peptide, enabling exosome targeting capability to cells bearing a matching receptor (Xitong & Xiaorong 2016). This modification is relatively uncomplicated since the targeting ligands can be genetically fused to the extra-exosomal termini of exosomal membrane proteins using PCR-techniques, without affecting the stability or biochemical properties of the exosome itself (Hung & Leonard 2015).

3.1.2.2.1 Commonly used transmembrane proteins for fusion with targeting ligands

In theory any transmembrane protein, if enriched in exosomes during biogenesis, can be used for fusion with a targeting ligand (Xitong & Xiaorong 2016). Nevertheless, Lactadherin, lysosome-associated membrane protein-2b (Lamp-2b) and Platelet-derived growth factors (PDGFRs) are three transmembrane proteins commonly used in exosome targeting (Hung & Leonard 2015, Xitong & Xiaorong 2016). It appears that lysosome-associated membrane protein-2b (Lamp-2b) is best suited for display of a targeting ligand (Hung & Leonard 2015). The protein has proven successful in enhancing specific delivery to the brain, neurons (Alvarez-Erviti *et al.* 2011), breast cancer cells (Tian *et al.* 2014) and cardiomyocytes (Mentkowski & Lang 2019) without any major complications or challenges.

3.1.2.2.2 Commonly used targeting peptides and their targets

To enhance targeting and drug delivery to the brain, two feasible options are addition of a brain-specific rabies viral glycoprotein (RVG) or an internalizing RGD peptide to the N-terminus of Lamp-2b (Alvarez-Erviti *et al.* 2011). Fusion of a differently derived RVG-protein to the same terminus of Lamp-2b resulted in exosome uptake via the nicotinic acetylcholine receptor, leading to enhanced drug delivery to neurons (Hung & Leonard 2015). Fusion of the internalizing RGD peptide and Lamp-2b has also been used to target αvβ3 integrins, enhancing exosome uptake to breast cancer cells (Tian *et al.* 2014). By engineering cardiosphere-derived cells to express Lamp-2b fused with a cardiomyocyte specific peptide (CMP), researchers have been able to enhance drug delivery to cardiomyocytes (Mentkowski & Lang 2019). These targeting ligands are fused onto the exosomal surface before isolation and purification of the vesicle. Therefore it is of great importance to choose methods that during production does not affect these targeting ligands in a way that decreases target specificity.

3.1.3 Challenges using targeting peptides to ensure target specificity

Different challenges can arise when relying on a specific surface molecule to achieve targeted delivery to desired cell type or tissue. Many exosomal components have not yet been identified or characterized, and how these components interact and interfere with other cell types in the host cells is unknown (Kibria *et al.* 2018). These unwanted interactions can affect how the targeting ligand interacts with recipient cells, complicating the delivery process.

A challenge with using targeting peptides fused to the N-terminus of transmembrane proteins is that these

peptides are exposed to extra-exosomal environments (Xitong & Xiaorong 2016). Therefore, these peptides are prone to degrade rapidly *in vivo*, resulting in a loss of targeting capability. Hung & Leonard (2015) proved that addition of a glycosylation peptide motif to the N-terminus of the targeting peptide protects the peptide from degradation. The modification did not affect interactions between targeting peptides and their targets, but increased the expression of the targeting peptide in native cells as well as exosomes derived from these, which further enhanced the delivery of therapeutic cargo to targeted cells.

3.1.4 Which modifications produce the most optimal drug delivery vehicle?

Despite the ability to deliver its cargo to recipient cells, some modifications of the exosome is required to ensure delivery to intended targets whilst minimizing unwanted delivery to other tissues and organs. It is of great importance to take these modifications into consideration when producing exosomes to use as drug delivery vehicles, since some isolation- and purification methods might interfere with components enabling specific targeted delivery.

To achieve an increase of target specificity, naturally occurring surface molecules can be enriched by inducing overexpression of these molecules in donor cells, or a targeting ligand can be fused to transmembrane proteins enriched in the exosome. One of the most promising modifications appears to be fusion of targeting ligands to the transmembrane protein Lamp-2b. Choice of targeting ligand depends on desired recipient cells, but it is common to use RVG-peptides and internalizing RGD peptides to target brain, neurons or cancer tissue. To avoid degradation of targeting ligands and further enhance the delivery, addition of a glycosylation peptide motif to the N-terminus of targeting peptide is a promising option.

3.2 Cargo loading

To be able to make use of exosomes in the context of targeted drug delivery there need to be a way to load specific cargo into them. This specific cargo can range from proteins (Haney *et al.* 2015) to nucleic acids (Alvarez-Erviti *et al.* 2011) and other small molecules (Kalani *et al.* 2016). There are many things to consider when discussing exosome cargo loading methods. Cargo needs to be loaded into exosomes while still remaining biochemically potent. The exosomes also needs to be undamaged by the method. Furthermore, a methods simplicity and cost also plays a role in how useful it is. In general when dealing with exosome loading it is always vital to consider what is being loaded. Methods that works well in some instances may not be effective when dealing with different cargo. For example electroporation is a widely used tool for exosome loading but is not well suited for loading longer nucleic acids such as DNA (Lamichhane *et al.* 2015). Overall many methods have been suggested and tried, some of the most widely used are passive diffusion through simple incubation, electroporation, sonication and transfection of exosome releasing cells, see Figure 4. A summary of loading methods can be found in Table 2.

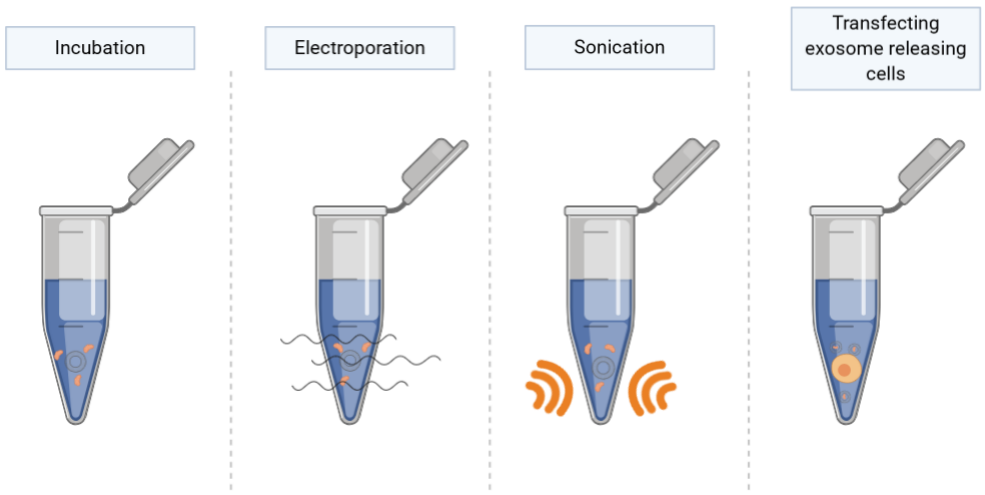

Figure 4. Illustration of passive diffusion thorough incubation, electroporation, sonication and transfection of exosome releasing cells. All used methods for incorporating different cargo into exosomes.

3.2.1 Protein loading

Many diseases are caused by the lack of or dysfunction of certain proteins. This fact makes proteins play a large role in various treatments (Dimitrov 2012). However, many proteins are only stable during specific conditions which often makes treatment difficult if not unviable. Exosomes ability to safely store and transport these proteins provides a solution, as long as there exists ways to load proteins into exosomes which does not interfere with the proteins function. Due to proteins size, protein loading into exosomes is a difficult task, although many techniques and approaches have been suggested and tried. The ones we evaluated as most promising will be discussed.

3.2.1.1 Exogenous protein loading

One approach to create exosomes loaded with specific proteins is to have a purified exosome medium mixed with the desired protein to be loaded. The mix is then exposed to the loading method of choice. A number of methods has been proposed and tried. Passive diffusion through incubation, freeze thaw-cycles, sonication, and extrusion have all been used to load exosomes with the enzyme catalase (Haney *et al.* 2015). In this study it was shown that especially sonication and extrusion were successful in loading exosomes with catalase. Freeze thaw-cycles resulted in an enzyme activity roughly half to that of sonication and extrusion while incubation resulted in an enzyme activity about an eighth to that of sonication and extrusion(Haney *et al.* 2015).

3.2.1.2 Endogenous protein loading

Another approach to exosome protein loading is to develop systems for loading exosomes with desired proteins in the cell producing the exosomes itself. For example a versatile system for modifying exosome composition was developed as early as 2005 when Delcayre *et al.* (2005) fused genes of interest to a specific region, the C1C2 region, of the gene coding for the exosome membrane protein lactadherin. Delcayre *et al.* (2005) called this system Exosome Display. The approach showed success and resulted in functionally active proteins on exosome

surfaces. The method has also seen use in other studies (Zeelenberg *et al.* 2008, Hartman *et al.* 2011). However this method loads proteins onto exosomes rather than into and as such is of limited potential as a drug delivery tool. The exosome display system seems to have most potential in purification and delivery applications.

For delivery into exosomes a method that shows more promise is the EXPLOR (Exosomes for Protein Loading via Optically Reversible Protein-Protein Interactions) system developed by Yim *et al.* (2016). The system consists of fusing the desired protein to be loaded with the photoreceptor cryptochrome 2. When exposed to blue light cryptochrome 2 binds to the CRY-interacting protein CIBN which in the system is conjugated to the exosome membrane protein CD9. This allows the desired protein to be incorporated into the exosome during its biogenesis. When the light is switched off this results in the protein being unattached from the membrane inside the exosome. The study showed that this system successfully encapsulated fluorescent target proteins in exosomes. This system is illustrated in Figure 5.

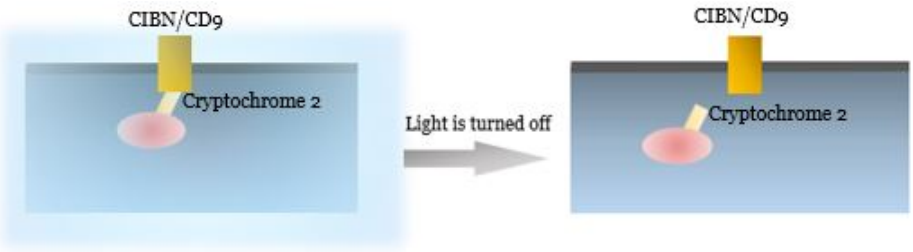

Figure 5. Illustrating the EXPLOR system used by Yim et al. (2016). In the picture to the left the photoreceptor is exposed to blue light and thus stays attached to the exosome membrane wall. When this light is turn off the protein is released into the exosome.

Another system for protein loading that has shown promise is the XPACK (Exosome membrane-anchored protein) system developed by System-Biosciences (2020).

3.2.2 Nucleic acid loading

Naïve exosomes, i.e. unmodified exosomes, have been shown to contain DNA (Thakur *et al.* 2014, Cai *et al.* 2013), mRNA (Li *et al.* 2014), miRNA (Li *et al.* 2014, Jia *et al.* 2018), tRNA (Li *et al.* 2014), and rRNA (Li *et al.* 2014) as well as a number of other types of RNA (Li *et al.* 2014). The fact that exosomes naturally host these nucleic acid makes them suitable as a transportation device for nucleic acids in medicine. A number of studies have been performed where exosomes were loaded with a cargo consisting of siRNA and miRNA. Electroporation (Alvarez-Erviti *et al.* 2011, Faruqu *et al.* 2018, Wahlgren *et al.* 2012, Yang *et al.* 2017), chemical transfection (Shtam *et al.* 2013, Wahlgren *et al.* 2012) and sonication (Lamichhane *et al.* 2016) have been used to exogenously load exosomes with short nucleic acids. Of these, electroporation has been the most widely applied method. Endogenous methods have also been used to load nucleic acids where exosome producing cells are transfected with miRNA which is then incorporated into exosomes (Akao *et al.* 2011).

3.2.2.1 siRNA loading

A study by Alvarez-Erviti *et al.* (2011) successfully used electroporation to load exosomes with siRNA with the aim of lowering the amount of the protein BACE1 in neurons. A 60 % mRNA knockdown was observed which shows that the exosomes were successfully loaded with the siRNA and delivered. However, when a study by Ohno *et al.* (2013) tried to use the same protocol to load exosomes they were unsuccessful. Ohno *et al.* (2013) hypothesized this was due to the different cell types used in the two studies. The strange thing about this is that Faruqu *et al.* (2018) used the same protocol as Alvarez-Erviti *et al.* (2011) and the same cell type (HEK-293) as Ohno *et al.* (2013) and was successful in using electroporation to load exosomes with siRNA with a fluorescent marker.

A study by Shtam *et al.* (2013) used chemical loading to load siRNA labeled with a fluorescent marker. This chemical loading consisted of mixing siRNA with the transfecting agent lipofectamine. This forms lipid complexes containing the siRNA and these were then mixed with exosomes and incubated for 30 minutes. Afterwards molecules not attached to exosomes were removed from the mix. These exosomes were then incubated with the recipient cells for 24 hours. The study showed that the siRNA was effectively delivered into the cells, however it could not say if it was the actual exosomes or lipid complexes attached to exosomes that stood for the delivery.

Wahlgren *et al.* (2012) used both electroporation and chemical transfection to incorporate siRNA tagged with a fluorescent marker into exosomes. Chemical transfection was performed using HiPerFect transfection reagent and then incubating. However, Wahlgren *et al.* (2012) later experienced the same issue as Shtam *et al.* (2013) where they could not confirm that the delivered siRNA in cells came from the exosomes and not the lipid complexes formed by the transfection agent. This was not an issue when using electroporation. Overall the study showed, similarly to the studies conducted by Alvarez-Erviti *et al.* (2011) and Faruqu *et al.* (2018) that exosomes loaded with siRNA using electroporation effectively can deliver potent siRNA to cells.

Apart from electroporation and chemical transfection sonication has also been used to incorporate siRNA into exosomes (Lamichhane *et al.* 2016). The study from 2016 examined sonication's potential as an alternative to electroporation as a loading method and found that the two alternatives were both viable with comparable rates of loading efficiency.

3.2.2.2 miRNA loading

In 2017 electroporation was used to successfully load RVG-modified exosomes with miRNA (Yang *et al.* 2017).

A study from 2017 used the novel approach of calcium chloride transfection together with freezing and heat shocks as a loading method (Zhang *et al.* 2017). The study also used electroporation for loading with the objective to compare it to its $CaCl_2$-based method and found that the two loading techniques had similar loading efficiencies. This speaks in favor of calcium chloride transfection since it does not require advanced equipment like electroporation does.

Another approach to miRNA loading have been to transfect exosome releasing cells with miRNA (Akao *et al.* 2011). These cells will then release exosomes containing this cargo and thus the miRNA loading is complete.

This approach has been shown to be effective and has been used to successfully load exosomes a number of times (Akao *et al.* 2011, Ohno *et al.* 2013, Katakowski *et al.* 2013, Liu *et al.* 2017, Munoz *et al.* 2013, Lou *et al.* 2015, Wang *et al.* 2016, Su *et al.* 2016).

3.2.2.3 Long nucleic acids loading

mRNA loading into exosomes require more intricate and delicate loading methods than short RNA segments. In a study from 2018 mRNA was loaded into exosomes endogenously by attaching a C/D-box RNA structure to the mRNA that was to be loaded (Kojima *et al.* 2018). This C/D-box structure is known to bind to the protein L7Ae which was in turn conjugated to the exosome membrane protein CD63, the complex is shown in Figure 6. The exosome loading of mRNA using this method was successful and the study showed that all parts of the loading complex needed to be in place for the exosome loading to be successful.

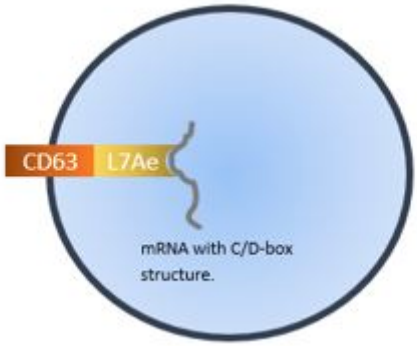

Figure 6. Schematic sketch over the mRNA loading complex used by Kojima et al. (2018).

Electroporation has been shown to be unsuccessful in loading long DNA strands (over 250 base pairs) (Lamichhane *et al.* 2015). However in a study from 2016 short (25 base pairs in length), single strand DNA was incorporated into exosomes using electroporation (Lamichhane *et al.* 2016). In this study sonication was also successfully used to load exosomes with single strand DNA.

3.2.3 Small molecules loading

Exosomes ability to transport molecules extends further than to biomolecules usually found in exosomes such as RNAs and proteins. Many studies have, for example, loaded exosomes with small molecules used to combat cancer cells (Sun *et al.* 2010, Tian *et al.* 2014, Hadla *et al.* 2016, Smyth *et al.* 2015, Kim *et al.* 2018, Bellavia *et al.* 2017, Tian *et al.* 2018b, Zhuang *et al.* 2011, Iessi *et al.* 2017). To load these molecules the methods that have been tried are passive diffusion through incubation (Sun *et al.* 2010, Bellavia *et al.* 2017, Tian *et al.* 2018b, Zhuang *et al.* 2011, Iessi *et al.* 2017), electroporation (Tian *et al.* 2014, Hadla *et al.* 2016, Smyth *et al.* 2015), and sonication (Kim *et al.* 2018).

Passive diffusion is a simple loading method that can be used to load hydrophobic drugs. Sun *et al.* (2010) used incubation and mixing to incorporate the polyphenol curcumin into exosomes. The study showed that curcumin

was successfully encapsulated using incubation and remained biochemically potent. This very same method to load curcumin has also been used in a study by Zhuang *et al.* (2011) as well as a study by Tian *et al.* (2018b). Passive diffusion has also been used to load exosomes with other drugs used in cancer treatment such as imatinib (Bellavia *et al.* 2017), acridine orange (Iessi *et al.* 2017), celastrol (Aqil *et al.* 2016), withaferin A (Munagala *et al.* 2016) as well as anthocyanidins (Munagala *et al.* 2016).

Three studies by Tian *et al.* (2014), Smyth *et al.* (2015), Hadla *et al.* (2016) all loaded exosomes with doxorubicin which is a chemotherapy drug used to treat cancer (Denard *et al.* 2012). Both Tian *et al.* (2014) and Hadla *et al.* (2016) effectively used electroporation to load exosomes with doxorubicin. Smyth *et al.* (2015) used passive diffusion as a loading method which also successfully loaded exosomes with doxorubicin. It is however difficult to compare the loading methods between the studies since they used varying amounts of exosomes, doxorubicin, and incubation time. Furthermore not all studies directly calculated how much doxorubicin was actually loaded. Overall both electroporation and passive diffusion seems to be viable methods of doxorubicin loading.

Kim *et al.* (2018) used sonication to load exosomes with paclitaxel, a chemotherapy drug (Lück & Roché 2002). Sonication was followed up with 60 minutes incubation to allow the exosome membranes to stabilize. This proved to be an effective loading method.

3.2.4 Table over loading techniques

Table 2. Summary of exosome loading methods.

Loading method	Cargo loaded	Comments
Passive diffusion	• Curcumin • Imatinib • Acridine orange • Celastrol • Withaferin A • Anthocyanidins • Catalase (poor loading. More effective when combined with freeze/thaw cycles)	As simple and cheap as is possible for a loading technique. Has limitations on when it can be used.
Electroporation	• miRNA • siRNA • ssDNA • Doxorubicin	Relatively simple method with effective loading that is well suited for many small molecules. Can however cause exosome aggregations (Johnsen *et al.* 2016).
Sonication	• Paclitaxel • siRNA • Catalase	Relatively simple method that suits well for some molecules. Can unlike electroporation be usd to load proteins. Can cause exosome aggregations.
Extrusion	• Catalase	Relatively simple method that can be used to load proteins.
Chemical transfection	• siRNA • miRNA	Simple method that does not require expensive equipment like for example electroporation. Varying effectiveness.
Transfecting exosome releasing cells	• miRNA	Effective method. Not very well understood.
Endogenous loading systems	• Proteins • mRNA	Complex systems. Have potential to load larger and more complex molecules.

4 Exosome structure and its importance to drug delivery

In order to produce exosomes for targeted drug delivery, the exosomes must be isolated and purified and the yield and composition analyzed. Many isolation- and detection-methods are based on exosomal features such as size, density, shape, surface charge and biochemical composition. The inherent targeting capability is also dependent on exosomal composition. Thus, it is of great importance to understand how the structure can be utilized for development of exosome-based drug delivery systems. In this section a description of common structural components of exosomes are provided, as well as an investigation of how these structural components can be utilized for isolation, detection as well as targeted delivery. All discussed structural components are illustrated in Figure 7.

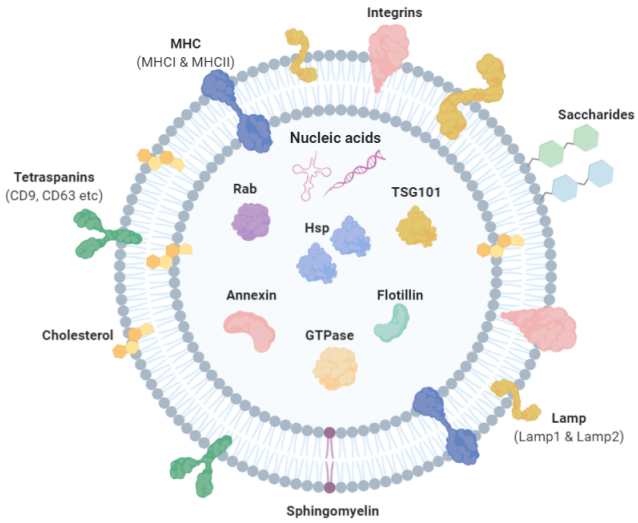

Figure 7. Schematic illustration of an exosome. Exosomes are composed of different lipids (sphingomyelin and cholesterol), proteins (tetraspanins, MHC, integrins, Lamp, Rab, Hsp, annexin, GTPase, flotillin, TSG101), saccharides and nucleic acids. The specific composition depends on the cellular origin of the exosome (Frydrychowicz et al. 2015).

4.1 Proteins are a prerequisite for isolation, analysis and targeted delivery

Exosomes contain a vast variety of proteins, many originating from the parent cells (Ha *et al.* 2016). Because of their endosomal origin, exosomes contain proteins involved in multivesicular biogenesis, heat shock proteins, tetraspanins, membrane transport proteins and membrane fusion proteins (Vlassov *et al.* 2012). Other proteins associated to exosomes are incorporated during exosome formation, serving as cargo during cell-cell communication (Ha *et al.* 2016). The proteins are commonly used as positive markers to detect exosomal

presence, and can also be utilized for isolation of exosomes using affinity- or antibody based techniques such as Western Blot or Enzyme-Linked Immunosorbent Assay (ELISA) (Vlassov *et al.* 2012, Ha *et al.* 2016).

4.1.1 Surface proteins enable recognition and targeting capability

Transmembrane proteins commonly enriched in exosomes are tetraspanins such as CD9, CD63, CD81 and CD82, integrins, major histocompatibility complex class I and II proteins (Conde-Vancells *et al.* 2008) and lysosomal proteins such as Lamp-2b (Caby *et al.* 2005). Tetraspanins are extensively used as positive markers for exosomal presence (Johnsen *et al.* 2014), for specific isolation using affinity purification with antibodies to these proteins (Vlassov *et al.* 2012), as well as for targeted delivery of exosomal cargo (Rana *et al.* 2012). Other transmembrane proteins commonly used as markers for detecting exosomes are integrins (Ha *et al.* 2016) and major histocompatibility complex (MHC) proteins (Frydrychowicz *et al.* 2015). MHC proteins can also be utilized for specific targeted drug delivery to T-cells (Johnsen *et al.* 2014).

4.1.2 Cytosolic proteins can be utilized for isolation and detection

Cytosolic proteins commonly found in exosomes include membrane transport and fusion proteins such as GTPases, annexins and flotillin, heat shock proteins such as Hsc70 and Hsp90, proteins involved in multivesicular biogenesis such as alix and TSG101, and Ras-related proteins (Conde-Vancells *et al.* 2008, Subra *et al.* 2010). These cytosolic proteins are used as markers for detection of exosomes as well as for isolation of exosomes using affinity purification (Vlassov *et al.* 2012).

4.2 Saccharides enable specific isolation of exosomes

The surface of exosomes is enriched with saccharides such as mannose, polylactosamine, α-2,6 sialic acid and complex N-linked glycans (Batista *et al.* 2011). In a strategy applied by Aethlon-Medical-Inc (2020), these saccharide residues are used to specifically isolate exosomes using affinity-capture methods using lectins with high affinity to saccharide residues on the surface of exosomes.

4.3 Nucleic acids for detection of exosomal presence

The cytosol of exosomes are found to contain nucleic acids, such as DNA, miRNA, mRNA, rRNA, tRNA, snRNA and other non-coding RNA species (Li *et al.* 2014). A potential marker for exosomal presence is miRNA (Vlassov *et al.* 2012), since the composition of miRNA in exosomes are significantly different from the composition in parent cells or other microvesicles (Mittelbrunn *et al.* 2011, Zomer *et al.* 2010).

5 Lipid nanoparticles as drug delivery vehicles

Lipid nanoparticles (LNP) are interesting as drug delivery systems for many reasons. The ability to carry lipophilic and hydrophilic drugs to specific cells or tissues without the disadvantage of being toxic is much needed to treat diseases. Both solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) will be considered in the report. Further, structural improvements for increased stability and specific targeting will be discussed.

5.1 The benefits of using lipid nanoparticles in drug delivery

Encapsulated drugs in synthetic made LNP have multiple advantages compared to equivalent free-drugs. An orally administered non-enclosed drug with low solubility in the gastrointestinal tract and low permeability across cell membranes limits its biological application (Ganesan & Narayanasamy 2017). According to Shah *et al.* (2015), an estimated 70 % of the new drug discoveries have issues with low solubility. Moreover, roughly 40 % of the immediate-release oral drugs on the current market are essentially insoluble in water. The challenges regarding water-insoluble drugs include poor bioavailability, precipitation after intake and finding a non-toxicological co-solvent (Shah *et al.* 2015).

The development of LNP have been demonstrated as a favorable strategy to elude the presented challenges with orally administered drugs and have the potential to enrich the pharmaceutical market as drug carriers (Shah *et al.* 2015). Drug size reduction, complexation, addition of surfactant solutions, development of pro-drugs and modifications of crystalline structures are strategies previously used to improve solubility (Shah *et al.* 2015). More control over drug release kinetics, more rigid morphology and more efficient entrapment of drugs are assets of LNP in contrast to previous developments of other lipid-based vesicles like liposomes and nanoemulsions (Puri *et al.* 2009). Shah *et al.* (2015) point out that LNP presents a viable biocompatible alternative and have proven to be a successful option with numerous benefits.

LNP are now an important part of green chemistry in the pharmaceutical industry for their biocompatible materials and the accessibility of environmentally friendly processes (Ganesan & Narayanasamy 2017). The lipids used to produce LNP are both biocompatible and biodegradable (Ganesan & Narayanasamy 2017). Thus, avoiding materials like polymers that can cause toxic effects during *in vivo* degradation.

LNP can efficiently increase the bioavailability of encapsulated drugs and facilitates pharmacological, toxicological and pharmacokinetic studies during drug development (Ganesan & Narayanasamy 2017). The advantages of LNP include improved mucosal adhesion and increased residence time in the gastrointestinal tract (Ganesan & Narayanasamy 2017). In addition, LNP can encapsulate hydrophilic as well as lipophilic drugs (Shah *et al.* 2015). The non-immunogenicity and non-toxicity aspects of using lipid-based particles as drug carriers facilitates the clinical application (Veiga *et al.* 2020). Shah *et al.* (2015) emphasizes the fact that LNP not

only reduces correlated side effects, but also optimizes the therapeutic benefits. Further, improvements in early diagnosis, prevention and control of disease are also considered to be facilitated by LNP. Overall, the development of nano-carriers has positively influenced the pharmaceutical industry with the production of uniform particles that have foreseeable release profiles that increase the therapeutic efficiency (Saghazadeh *et al.* 2018).

5.2 Synthetic nanoparticles with great potential as drug delivery vesicles

There are several types of lipid-based nanoparticles but in this report solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) are focused on. These two types of lipid nanoparticles are heavily studied and have desired features as appropriate stability and eminent drug loading capacity (Naseri *et al.* 2015). The rigid matrix in both SLN and NLC protects the enclosed drugs from degradation, modifies the biodistribution and assists in the alteration of the release profiles of the drug (Ganesan & Narayanasamy 2017).

5.2.1 Solid lipid nanoparticles as a synthetic made drug carrier

Solid lipid nanoparticles (SLN), solid in both room as well as internal body temperature is a much appreciated drug delivery vehicle (Naseri *et al.* 2015). The phospholipid coating around the solid core is made of fatty acids alternative versions of triglycerides (Mitra *et al.* 2017, Maeda *et al.* 2013). The average size of a solid lipid nanoparticle is 100-300 nm even though the size can be up to 1000 nm (Martins *et al.* 2007, Vitorino *et al.* 2011). SLN possess advantages as great storage stability, preferable bioavailability, low toxicity in its proper form as well as when degrading (Mitra *et al.* 2017, Maeda *et al.* 2013). The interest for SLN as a drug delivery system is due to the structural setup with the fatty-tails inwards, creating a safe space for hydrophobic and lipophilic drugs to be encased (Mitra *et al.* 2017, Mishra *et al.* 2018). Other characteristics making them appropriate is easy scale-up and low cost of production (Naseri *et al.* 2015). It is also notable to address that the preparation can be done without using organic solvents (Mohapatra *et al.* 2018). As a drug delivery system, intravenous use is most favorable as the hydrophilic characteristics is covering the outer part making it aqueous suitable (Mitra *et al.* 2017).

SLN has great prerequisites to be altered with modifications creating a design that allows specific targeting. This is done by adding ligands to the base structure, see more under Section 5.4.1. This together with the fact the SLN is naturally accumulating to tumor environments rather than normal tissue in a phenomenon called enhanced permeability and retention effect (EPR) makes SLN a well-suited particle for drug delivery in cancer therapeutic purpose (Maeda *et al.* 2013).

Notable complications with SLN is the immunoresponse and clearance by the mononuclear phagocyte system, and stability difficulties (Nienhaus *et al.* 2020). These will be addressed in the section about corona shields and polyethylene glycol (PEG) coating, see corona shield in Section 5.3.3 and PEG coating in Section 5.3.2.

5.2.2 Nanostructured lipid carriers for increased drug load capacity

Nanostructured lipid carriers (NLC) are looked upon as the next generation of SLN and allows a greater amount of drug loading due to its preparation mixture not only containing solid lipids but also liquid lipids (Puri *et al.* 2009, Beloqui *et al.* 2016, Müller *et al.* 2002). The drug load capacity allows, percentage by weight, 3-4 % and the internal structure is of a more imperfect character which enhance a greater load (Puri *et al.* 2009). The mixture of both solid and liquid lipids also generates a deformed crystal which besides granting better loading possibilities also increase stability and drug release prerequisites (Khosa *et al.* 2018). The size is in the same range as SLN, 100-500 nm (Martins *et al.* 2007) and it depends on the ratio of liquid and solid lipids (Khosa *et al.* 2018). It is usually 70:30 (solid:liquid) and it has been proved that up to 30 % liquid material, the particle size decrease when adding more of the liquid components (Khosa *et al.* 2018).

NLC deserves to be pointed out as an attractive future candidate on the therapeutic market due to two factors: i) the hydrophobic core allows both hydrophilic and lipophilic materials (Puri *et al.* 2009, Khan *et al.* 2015) and ii) to its ability to permeating the brain-blood barrier (BBB) (Khosa *et al.* 2018). The second advantage, to cross the capillaries in the brain is a stressed and since long time desired feature as 98 % of the new developed drugs are unable to penetrate to brain tissue (Khosa *et al.* 2018). At the same time diseases like Alzheimer, Parkinson, brain tumors are dependent on BBB permeable therapeutics (Khosa *et al.* 2018). To address the first point brought up, the ability to load different type of substances with repelling abilities, 40 % of all new produced drugs are of hydrophobic character (Martins *et al.* 2007). Therefore the demand of novel drug delivery systems is real. Further, NLC are easy to design and moderate with PEG and adapted ligands to increase the stability, the specific accumulation, increased internalization and drug release (Puri *et al.* 2009, Khosa *et al.* 2018, Martins *et al.* 2007). The benefits of PEG modification will be handled later in the report under Section 5.3.2.

The combination of high drug load capacity and great internalization, NLC treatments welcome a total drug reduction for patients in the future (Khosa *et al.* 2018). Since it first developed, in the late 1990's, methods of production have improved and the preparation of NLC is now done at a low cost and are able to be scaled up (Puri *et al.* 2009, Khosa *et al.* 2018).

Substances, compatible to add on the NLC surface allows controlled drug release following cellular uptake and the release can either be triggered by physical circumstances or natural lipid degradation (Martins *et al.* 2007). There is a keen understanding about NLC and how the modifications are effecting internal behavior due to easy absorption, through incubation, of image enabling substances (Martins *et al.* 2007).

5.2.3 Why SLN and NLC are excellent candidates for drug delivery

The two different nanoparticles' similar features include size, low toxicity and great bioavailability. Both SLN and NLC protects the enclosed drug, both lipophilic and hydrophilic, from degradation and are documented to be able to scale up and be produced to a low cost. Furthermore, both offers opportunity to be easily modified to improve targeting and stability. Both nanoparticles can utilize the EPR effect to be drawn to tumor tissues and also cross membranes. NLC is look upon as the next generation of SLN due to its further drug loading capability

and stability quality and future research should therefore focus on nanostructured lipid particles. The great amount of research done on SLN should be analyzed and examined when looking to improve the advanced NLC.

5.3 Structural components - modifications on designed nanoparticles for stability and prolonged circulation time

Synthetic drug delivery carries (DDC) provide local therapeutic support by passive or active targeting (Jokerst *et al.* 2011), Section 5.4. Both of the delivery methods rely on long circulation time and therefore must structural design modifications to uphold a stability be done (Mui *et al.* 2013). In this section, a handful of different modifications will be addressed.

5.3.1 Background - mononuclear phagocyte system

For the loaded lipid nanoparticles to accumulate and permit cellular uptake in the localized target area, it requires a prolonged circulation time without any macrophage interaction (Jokerst *et al.* 2011). For a successful cellular uptake, the drug carriers need to remain at a certain concentration (Jokerst *et al.* 2011). An immune response occurs when foreign substances or particles, like DDC, enters the body and cause accelerated blood clearance (ABC) (Zhang *et al.* 2016). Immunoproteins called opsonins binds the intruding item and macrophages reacts on the opsonin tags by removing it from the blood steam through endocytosis (Mui *et al.* 2013), see Figure 8. The mononuclear phagocyte system (MPS) implies macrophages engulfing non-self material and deliver it to either the spleen or the liver for degradation (Jokerst *et al.* 2011). Alterations on lipid nanoparticle's surface can be made and to occlude the binding sites for these immunoproteins.

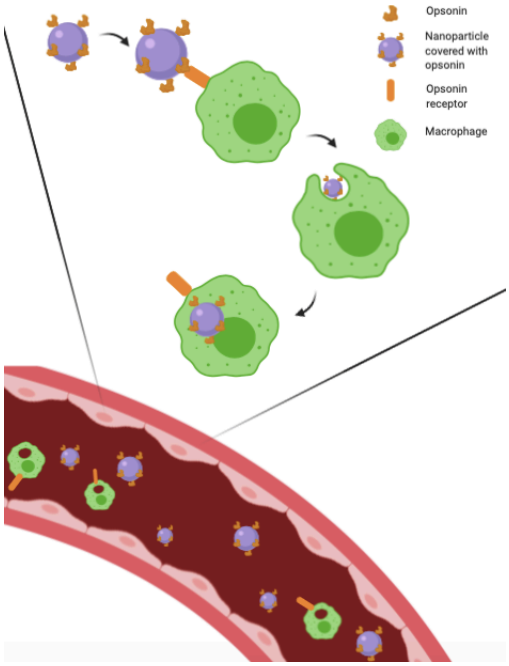

Figure 8. The immunoprotein opsonin binds to foreign items in the body and trigger macrophages to remove the marked objects from the blood stream by endocytosis. Macrophages have opsonin-receptors on the surface.

5.3.2 Modification with PEG - a molecule with many benefits

Polyethylene glycol (PEG) can be used in purpose of modification for 1. Act as a steric hinder for immunoproteins to avoid a macrophage clearance, 2. Reduce surface charge and then also diminish unwanted interactions, 3. Provide protection or act as an extended arm for better exposure for targeting ligands. Polyethylene glycol (PEG) is certainly one of the most commonly used modification additives for above mention purposes.

5.3.2.1 The molecule structure of PEG

The PEG molecule is composed of two ends with *n* numbers of ethylene glycol repeats in between as seen in Figure 9. One end is attached to the LNP surface, this one is refereed to as R1 and one is protruding the surface and is exposed to a secondary ligand, R2 (Eloy *et al.* 2017, Jokerst *et al.* 2011). The secondary ligand is tentatively an antibody (Eloy *et al.* 2017).

PEG

Figure 9. Polymerize of ethylene glycol, also refereed to as PEG. The R1 end is attached to the nanoparticle's surface and the terminus R2 end is available to further linkage. The length of the PEG may vary.

5.3.2.2 Physical alterations for improved circulation time

The length of the PEG molecule has shown to have an impact on circulation time. Mori *et al.* (1991) preformed a study of the effects of the PEG length on another kind of synthetic nanoparticles, liposomes, and found a relationship between an extended blood circulation time and increasing average molar mass of the PEG molecule (Mori *et al.* 1991). Even so, nanoparticles in the size range 50-100 nm are commonly coated with shorter kinds of PEG units due to that longer chains have shown shorter circulation time due to the increased hydrodynamic radius (Jokerst *et al.* 2011). The relation between size and successful result seems to not have a definite answer and depends on multiple variables. Therefor when preparing LNP, different attempts with alternating PEG arm lengths needs to be carried out and analyzed.

5.3.2.3 PEG decrease the surface charge and reduce unwanted interactions

One obstacle for well accomplished accumulation is the mutually nanoparticles interactions (Mui *et al.* 2013). By adding PEG to the surface, the surface energy is decreased which results in less van der Waals attraction and thereby less LNP-LNP interaction but also, PEG acts as a steric hinder for these unwelcomed interaction (Jokerst *et al.* 2011). Further, the phagocyte's negative cell surface relies on opsonins to reduce the negative charge on intruding biomatter or particles to reduce the repulsion in-between phagocytes and the particles (Jokerst *et al.* 2011). Therefore, modifying the nanoparticles surface with negative ends-groups will increase the repulsion to macrophages and create a MPS stealth effect. This is done by adapting PEG with an end group of usually thiol or carboxyl (Jokerst *et al.* 2011).

5.3.2.4 PEG modification for specific targeting and stability

The desire with LNP is to initiate greater affinity for a targeting recipient cell and PEG allows this by helping targeting ligands to either attach on the molecule or to protect the surface-connected ligand. The terminal end of the PEG molecule is exposed to a secondary ligand and the advantage of not attaching an antibody directly to the LNP surface is the result of well orientated antibody conformation (Eloy *et al.* 2017). To attach antibodies on a PEG-arm have been associated with an exposure of the antigen epitope which triggers an immunoresponse (Zhang *et al.* 2016, Jokerst *et al.* 2011). Therefore, the surface is often designed with a mixture of PEG-chain

lengths. Shorter ones for stability and longer for secondary ligands (Jokerst *et al.* 2011). Another approach to create stability but low MPS interaction is to attach the ligands on the surface and cover the rest of the LNP with PEG to protect epitope recognition (Jokerst *et al.* 2011). Even though the last mentioned approach creates inaccessible ligands and therefore lower rate of site-specific accumulation this could be a solution (Jokerst *et al.* 2011). The different types of composition are illustrated in Figure 10. There is a balance-based problem to find effective proportions of PEG and ligand exposure to obtain low immunogenicity, high specific binding and accumulation and great colloidal stability (Oh *et al.* 2018, Yoo *et al.* 2019, Zhang *et al.* 2016, Jokerst *et al.* 2011).

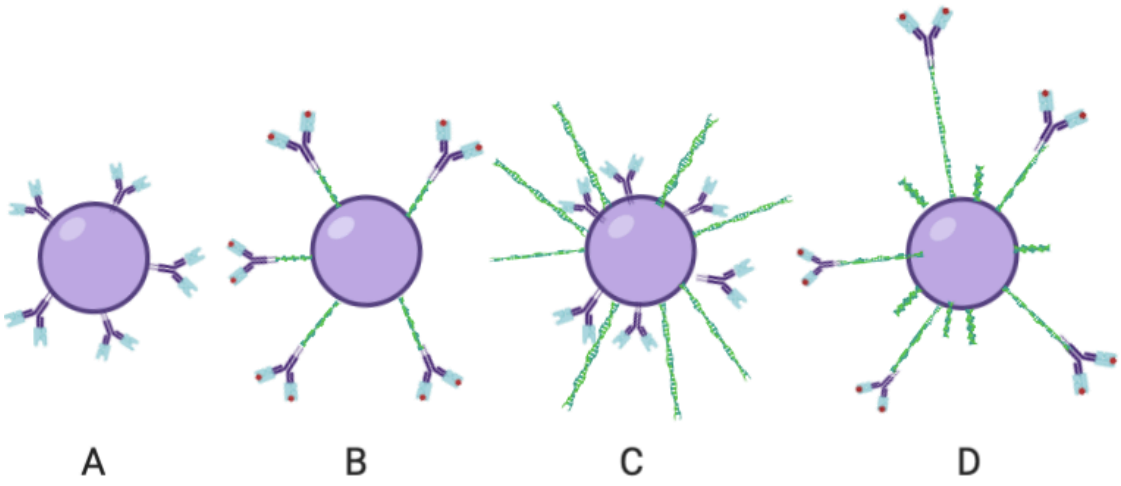

Figure 10. Illustration of a modified nanoparticle with ligands. (A) show ligands attached directly to the surface, (B) show ligands attached to a PEG arm, (C) illustrates surface-attached ligands protected by long PEG chains and (D) demonstrates a mixture: short PEG to stabilize and long PEG for ligand attachments.

5.3.2.5 PEG should be considered as an important modification on LNP

There is great support that PEG units increase blood circulation time *in vivo* (Suk *et al.* 2016, Tan *et al.* 1983) and an early study represented results of a 40-times increased circulation time between a non-coated and a coated particle (Tan *et al.* 1993). The ability to act as both a physical and a hydrophilic steric hinder, avert macrophage clearance and increase the totalt stability makes PEG an unique co-surfacant. Therefore should modification with PEG be considered when looking for designing a successful lipid nanoparticle.

5.3.3 Protein corona on nanoparticles – an obstacle and an opportunity

To further understand how to optimize the design of drug delivery systems one has to recognize the difference in *in vitro* and *in vivo* testing. A challenge only experienced in the natural biophysical environment inside the body, is the accumulation of blood circulating proteins around the LNP (Nienhaus *et al.* 2020). When LNP freely circulate in bloodstream natural collisions occurs and reversable binding interactions forms (Nienhaus *et al.* 2020). This adsorption of biomolecules creating an embracing layer is called a protein corona shield (Oh *et al.* 2018). The arrangement of physical changes are enthalpy driven and creates a more stable design (Nienhaus *et al.* 2020) and thereby reducing the surface energy (Oh *et al.* 2018). These changes typically modify the original structure and give the molecule a new identity (Nienhaus *et al.* 2020). The altered structure can enforce

further interactions with other LNP or macromolecules and interfere with the initial purpose of target accumulation. Examples of interactions is van der Waals, covalent, electrostatic and hydrogen bonds. Examples of interactions is van der Waals, covalent, electrostatic and hydrogen bonds. Oh *et al.* (2018) explains that one possible explanation why few synthetic made nanoparticles with drug cargo has passed clinical trials is due to the original aim of specific targeting ability is reduced when the cloak of biomolecules is masking the surface ligands (Oh *et al.* 2018). This factor is addressing the already mentioned problem of accumulation and low degree of internalization (Oh *et al.* 2018).

5.3.3.1 Blood circulation promotes naturally occurring corona shields

To create a drug delivery vehicle that will remain pristine it is crucial to understand the process of protein corona formation and how to regulate it. First of all, one needs to acknowledge that blood serum contains over 3,700 identified proteins and to predict the different variations of layer configuration is not possible (Docter *et al.* 2015). Nienhaus *et al.* (2020) writes that after introduced to serum biofluids the nanoparticle has absorbed up to a few hundreds of proteins (Nienhaus *et al.* 2020). Second, the composition of the protein corona also depends on the lipids structure and components as well as the adjacent microenvironment (pH, temperature, concentration etc.) making the prediction process even more difficult (Nienhaus *et al.* 2020). Therefore, other approaches like further customization of the design of the LNP further to be more resistant to the changes colloidal entail, are far more relevant.

5.3.3.2 Lab-engineered pre-coated corona particles for protection of ligands and immune response

By approaching the problem through a lab- moderated pre-coating process the LNP can achieve both chemical and physical stability (Nienhaus *et al.* 2020). The pre-coated shield induce protein aversion and leaves the LNP intact and possible targeting ligands free to bond with the matching receptor (Nienhaus *et al.* 2020). The protein aversion that applies to pre-coated LNP that is mentioned above, also results in repulsion of opsonins, immuneresponse-calling proteins (Jokerst *et al.* 2011). As opsonin is a protein recruiting mononuclear phagocyte one of the most successful approaches to hindrance the affiliation between opsonin and LNP is to decrease the affinity by coating the particle with great hydrophobic parts (Cui *et al.* 2018), see Figure 11. The phenomenon of abscond the mononuclear phagocyte system, MPS, and increasing the circulation time is called the stealth effect (Nienhaus *et al.* 2020).

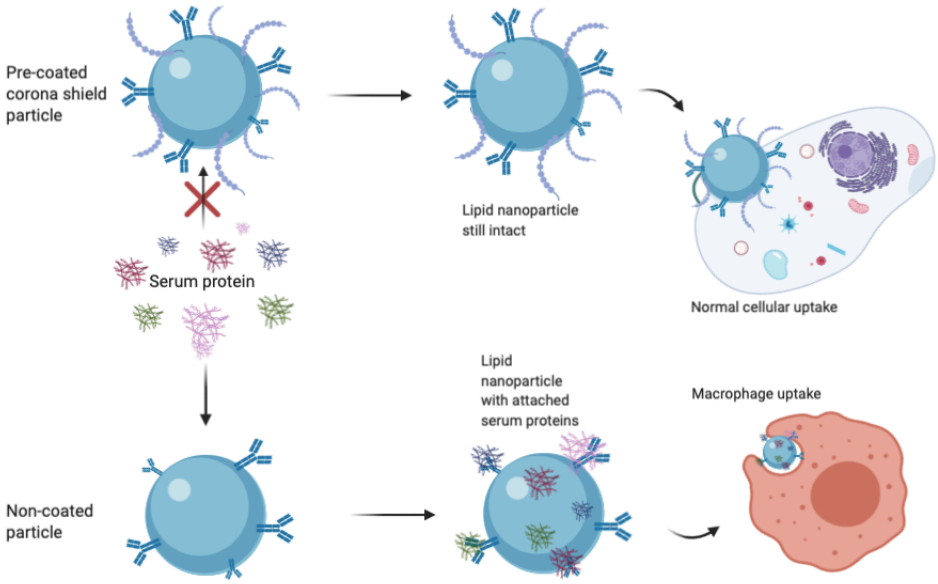

Figure 11. Illustration of how pre-coated lipid nanoparticles prohibits serum proteins to bind and trigger immunrespons.

5.3.3.3 PEG - a well-known candidate for lipid nanoparticle modification

Even if there are many alternatives to use as corona constituent, when designing LNP, it is desirable to use molecules that may contribute with many benefits. The PEG molecule is commonly used as mention in the previous section, 5.3.2, and has in many studies showed to increase LNP stability (Jokerst *et al.* 2011). PEG does not only contribute to sterically occlude other biomatter to interfere with the LNP surface, but also amplifies the hydrophilic properties which importantly induce protein aversion (Nienhaus *et al.* 2020). Therefore are PEG is a preferable candidate as a corona component (Nienhaus *et al.* 2020).

5.3.3.4 Corona is not only and obstacle. It's an opportunity

As the paper by Nienhaus *et al.* (2020) states "protein corona is not be considered an obstacle, but rather an opportunity to achieve selective biological responses…". The opportunity to enhance the targeting specificity and increased accumulation due to pre-coating should be acknowledged. By adding proteins suppressing the phagocytic attention one could increase the stealth effect (Nienhaus *et al.* 2020), see Section 5.3.4. As PEG has proven to be a useful modification molecule for other purposes, brought up in /refPEG, it seems most convenient to consider PEG as a corona component as well, leaving the molecule to serve for multiply services and reduce further modification steps.

5.3.4 Leukosomes - biomimetic to avoid leukocytes and improve biological acceptance

In targeted drug delivery, disease specific conditions are requested when looking at the accumulation factors to ensure guaranteed definite delivery (Devarajan *et al.* 2015). Above was an example of biomimetic when designing for improvements. Additional attempts of biomimetic have been done when incorporation membrane proteins of leukocytes onto liposomes, another lipid-based particle (Martinez *et al.* 2018). This new merged

particle group is called leukosomes (Martinez *et al.* 2018). Beside upholding the stealth effect to avoid the mononuclear phagocyte system there is also of interest to avoid leukocytes. They are the first responders to inflammation in the body (Chan *et al.* 2010). Inflammation is the body's response to early stages of diseases and on the cue the endothelium express vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Martinez *et al.* 2018).

Therefore are the endothelium and the overexpressed VCAM-1 and ICAM-1 are a promising system for designed immunoliposomes. The mimetic is done by adding the proteins of the leukosomes membrane to provide a "markers of self" effect and counteract the phagocytosis procedure (Martinez *et al.* 2018, Yoo *et al.* 2019).

These leukosomes are based on 140-170 nm spherical sized liposomes with membrane integrated cholesterol as stabilizers (Martinez *et al.* 2018), see more in Section 5.3.5. Martinez *et al.* (2018) describes an experiment they performed treating mice with developed 4T1 tumors with control liposomes and modified leukosomes (Martinez *et al.* 2018). This tumor progression triggered an inflammatory surrounding inviting leukosomes to accumulate to a greater extent than liposomes as these lacks the "self" tag. By fluorescence based imaging the result from the experiment showed 16 times less accumulation and internalization for regular liposomes. Further analyzing presented that leukosomes was present up to 9 hours after treatment to 15 times greater in concentration (Martinez *et al.* 2018).

The two protein ligands that were identified, after further research, to be significant in targeting is α-CD45 and LFA-1 (Martinez *et al.* 2018). These added attributes on the lipid structure seemed to increase the avoidance of MPS.

5.3.5 Cholesterol

When aiming to further stabilize a drug delivery carrier (DDC) one has to consider the immune response every additional adjustment might trigger and thus enhance rapidly clearance by the MPS. It is expected that biomaterials already existing in the body would induce low toxicity response when integrating with other non- or low toxicity material (Lee *et al.* 2016). Cholesterol is the most common sterol in mammals and is a natural occurring unit in membranes (Kohli *et al.* 2014). Suñé-Pou *et al.* (2018) designed a solid lipid nanoparticle and incorporated cholesterol in the membrane structure with the aim to improve cellular uptake and at the same time not be toxic to the targeted cell for efficient nuclear acid therapeutic (Suñé-Pou *et al.* 2018). The experiment reported that cholesterol integrated in the SLN increased the internalization of the cells as enhanced bioactivity was observed (Suñé-Pou *et al.* 2018).

5.4 Structural components - modifications for specific targeting

As mentioned, lipid-based nanoparticles possess the advantage of being able to deliver drugs otherwise too hydrophilic and with too low water solubility for other delivery systems (Puri *et al.* 2009, Khan *et al.* 2015).

Improvements of the design is constantly explored to address the challenges of accumulation and internalization, but also internal degradation and internal drug dispense. Modifications and additives to the surfaces are often included when designing drug delivery vehicles for increased targeting. The sureface attached add-ons are called therapeutic ligands.

5.4.1 Surface attached ligands for increased accumulation and cellular uptake

In order to treat a variety of diseases, many of them with abnormal microenvironment, it is crucial for the delivery system to uphold a stability to be able to circulate until right area is located (Sapra & Allen 2003). To further overcome the problem of poor accumulation, surface modifications to improve specific targeting are explored and thought to be a promising improvement. After recognizing the targeted area and identifying unique features in the microenvironment these can with matching counterparts be adapted to the surface of the drug carrier to improve coupling of drug upon drug use (Sapra & Allen 2003). This way to access increased accumulation and internalization is called active targeting. The functionalized group added as the ligand is usually a protein, an antibody or affibody, a peptide, vitamin or carbohydrate. When no ligand is adapted, this is called passive targeting, see Figure 12.

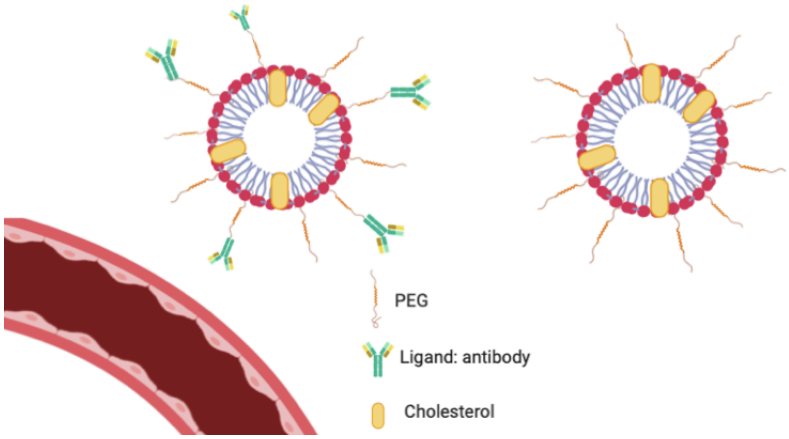

Figure 12. Active vs Passive targeting. Illustration of two lipid nanoparticles. The left one is modified with attached ligands, in this illustration: antibody. The right one is without ligand modifications.

5.4.2 Passive targeting requires less modification preparations but inducing a more general and less specific targeting

Passive targeting relies on enhanced permeability and retention, the EPR effect, which increase the half-life degradation (Kohli *et al.* 2014). A prolonged circulation is attained after adjustments of nanoparte surface like PEG, Section (5.3.2), corona shield, Section (5.3.3) and cholesterol, Section (5.3.5). These additives to the structure increase the stealth effect and allows the nanoparticle to avoid MPS and reduce risk of defects from collisions with serum proteins (Puri *et al.* 2009). In both inflamed and tumor areas, nanocarriers (NC) are known to assemble in higher concentration than in healthy tissue as the vasculature walls are fragile and damage leaving them leaking (Eloy *et al.* 2017). This phenomenon increases the permeability has been seen with the greatest

result when the size of liposome is between 100-200 nm (Noble *et al.* 2014).

Passive targeting therefore provides increased accumulation for designed and unmodified nanoparticles based on the physicochemical properties (Yoo *et al.* 2019). As passive targeting depends on EPR which is in many ways governed by factors like tumor size and status, shape and composition of NC, and unpredicted factors in the nearby surrounding, it is more ensuring to rely on active targeting (Puri *et al.* 2009).

5.4.3 Active targeting for specific delivery with opportunity to treat a variety of diseases

When considering a receptor or other docking partner for a ligand it is significant that this opponent is either tissue or disease specific. Many attempts on encouraged cellular uptake are done in cancer research and target tumor-specific receptors (Yoo *et al.* 2019, Attia *et al.* 2019, Leamon & Low 2001, Greish 2012, Smith *et al.* 2011). In active targeting it is the headgroup, the hydrophilic head on the lipid, that is modified with ligands and the specific delivery relays on headgroup interactions with the tissue or cellular type in question (Kohli *et al.* 2014). The adapted ligands often interact with surface receptors on targeted cells and an increased concentration ligand on the surface has shown positive results in elevated uptake (Yoo *et al.* 2019). On the other hand, more decoration on the surface often enhance immune response. The balance of shielding the LNP for stealth effect and still decorate the surface with accessible ligands is fine tuning problem with great oscillating results (Dai *et al.* 2018). As mentioned above, even active targeting enjoys the privilege of EPR and the accumulation it confers (Attia *et al.* 2019). There is comprehensive material available on different attempts with variations of attached ligands, many with impressive results, so here there will only be one example of each of the following ligand groups: peptide, vitamin, affibody and antibody, Figure 13.

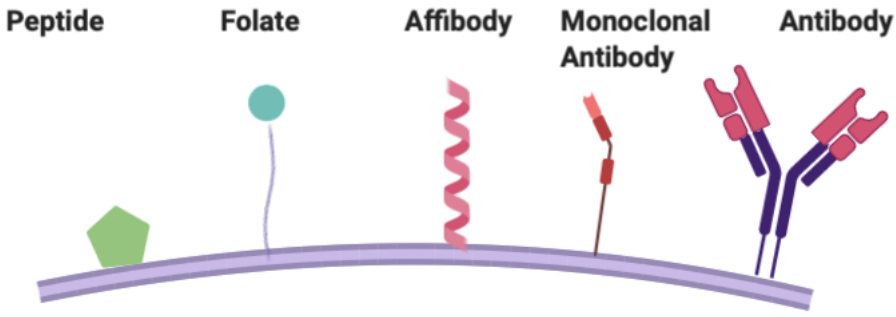

Figure 13. Ligands for active targeting. The figure demonstrates five different kinds of ligands that are used in specific targeting with lipid nanoparticles.

5.4.3.1 Peptide

Peptides are one of the studied groups of targeting additives of designed lipid-based nanoparticles. The tripeptide motif RGD, Arg-Gly-Asp, is of great interest by the cause that one third, eight out of 24, supertypes of intergrins is recoginzed by RGD (Schittenhelm *et al.* 2013). Integrins serves as transmembrane receptors and are important for internal, surrounding cells and the microenviorment (Nieberler *et al.* 2017). Integrins identify extracellular matrix (EMC) proteins through the RGD motif (Nieberler *et al.* 2017) and these integrins are highly

overrepresented in glioma cells as well in the tumor microenviorment (TME) especially on the blood vessels (Yoo *et al.* 2019, Attia *et al.* 2019). Triggered integrins in tumor areas have a roll in morphogenesis and growth (Schittenhelm *et al.* 2013). The integrin αvβ3 strongly recruit RGD and the docking have significant impact on cancer stem cells and encourages tumor growth (Nieberler *et al.* 2017). Therefore delivering suppressors and/or steric blocking substances packed in nanoparticles to these targeted sites with RGD ligands to increase accumulation beyond EPR is highly requested (Schittenhelm *et al.* 2013). In a successful try, Lu *et al.* (2018) demonstrated how, when adapting a RGD ligand on a packed nanoparticle, the cancer tumor managed to shrink (Lu *et al.* 2018). Peptides has proven to give positive results in different experiments and are a useful ligands as they are recognized by a variation of intergrins.

5.4.3.2 Vitamin

Vitamins has also showed to be promising ligands because many receptors are often overexpressed in cancer cells and tumor microenvironments. One of them are the vitamin folate receptor (FR) (Leamon & Low 2001). Therefor, using folate ligands as a targeting ligand in designed drug systems seems reasonable. The FR is present in many different types of cancer including ovarian, lung, kidney, breast and colon (Puri *et al.* 2009). Folate (FA) is low weighted vitamin, needed in eukaryotic cells for proper embryotic growth and one-carbon metabolism (Leamon & Low 2001). The fact that the concentration of FR is excessive in TME and also increases as the cancer state advance it is indeed an alluring ligand to consider in tumor therapeutic. The theory that an increased amount of receptors on cancer cells is linked with accelerated cell growth and mitosis is based on the observations that nearby cells with no cancer character does not possess the same receptor pattern (Toffoli *et al.* 1997).

Even though some studies suggested only modest results in an increased internalization, internal drug release and succeeding effects with FR targeting there is great hope for what engineering drug delivery particles can offer in near future (Leamon & Low 2001). As more knowledge about how nanoparticles needs to be composed to not sterically occlude essential components, the FA coated liposome advances in being bioavailable appropriate and therapeutic relevant (Leamon & Low 2001). Anu Puri *et al.* (2009) together with fellow researchers described a result of achieving a 45 times higher internalization and 85 times greater cytotoxicity result from when using loaded liposomes with FA targeting features (Puri *et al.* 2009).

The folate coupled ligands are described as efficient with future potential and should be considered in future investigations (Leamon & Low 2001). To use already body-accepted substances are a point of departure when looking for new ligands. The reduced immune response is desired and if the substance also has disease-specific receptors, one can embrace that the substance and consider it for modification designing.

5.4.3.3 Affibody

Features, as disease-correlated overexpressed receptor and no natural ligand to the receptor, are desirable when looking for novel candidate ligand counterparts. This due to the minimized risk of effecting healthy non-aiming cells and to abandon the competitive ligand-binding situation. Instead, lab engineered Affibody molecules are

made to access the receptor. Affibody molecules are designed proteins and are smaller, normal size is usually 6-9 kDa, and more stable than antibodies (Smith *et al.* 2011). The characteristic of having great affinity to their receptor is common for all Affibody molecules and are therefore highly interesting when modifying nanoparticles for therapeutic purpose (Smith *et al.* 2011). The HER2 receptor owns up to the previously mentioned desired features (Greish 2012, Smith *et al.* 2011). The receptor has been found in excess in aggressive breast cancer compared to as well normal breast tissue and other milder forms of breast cancer and with no natural ligand (Greish 2012). The receptor are involved in ligand dimerization with other receptors and promotes cell growth and prevent apoptosis (Greish 2012, Smith *et al.* 2011). The accumulation for the designed lipid nanocarrier with HER2-ligand increased and it resulted in an inhibited growth of the tumor by 90 % (Yoo *et al.* 2019, Smith *et al.* 2011).

It has been observed that Affibody molecules increase both the colloidal stability and the stealth effect which increase the blood circulation time (Yoo *et al.* 2019). This together with EPR and specific affinity by the ligands, there is an appreciated attraction to tumor cells (Yoo *et al.* 2019, Smith *et al.* 2011).

5.4.3.4 Antibody

The above-mentioned ligands are directed to target and integrate with a surface receptor that in damaged and tumor tissue show abnormal proportions and activity of these. The antigen ligand approach is based on surface antigens differences between healthy and other distinguishable cells (Rostami *et al.* 2014). Important desirable features in antibody (Ab) ligand design to bear in mind are that it is eligible for the receptor to be recycled, have an elevated receptor density and, obviously have a great antigen- antibody affinity (Puri *et al.* 2009). Antibodies are larger than affibodies and are up to tens of kDa (Yoo *et al.* 2019).

There is a contradictable conflict in the concentration of ligands. The greater the density of adapted ligands the greater is the immunoresponse but at the same time, the greater is the specific binding (Oh *et al.* 2018, Yoo *et al.* 2019). Even though the specific binding are in favor of multiply antibodys attached, the small surface area combined with the antibodies' great density prevents the designing lipid nanoparticles (LNP), to be enriched with too many (Yoo *et al.* 2019).

Monoclonal antibodies (mAb) are produced via B lymphocytes, a white blood cell vital for the immune system, which are fused with myeloma cells (Eloy *et al.* 2017). The mAb binds to epitope, also called the antigenic determent, and it is the region recognizing and matching with the antibody (Eloy *et al.* 2017). The structure of mAB consist of two antigen-binding fragments (Fab) joined by a disulfide bond (Kohli *et al.* 2014, Eloy *et al.* 2017). When the Fab's are disconnected they are excellent to attach on lipid nanoparticles (Kohli *et al.* 2014). The use of Fab's instead of mAb is the increased stability, great binding prerequisites but also the decreased MPS response (Kohli *et al.* 2014). This has otherwise been proved to be a problem (Oh *et al.* 2018, Dai *et al.* 2018). Additional coating of LNP and especially with ligands and without self-recognition factors has been observed to trigger attachment of opsonin (Nienhaus *et al.* 2020).

Not only is the antibody advantageously connected to the end of surface attached PEG molecule for the purpose

of controlled orientation of antibody to facilitate its recognition (Eloy *et al.* 2017), but also to aid transport across the blood-brain barrier (BBB) (Kuo & Ko 2013). This transmembrane obstacle has been identified as one of the most crucial bottlenecks in the use of lipid-based drug delivery carriers for therapeutic purpose (Kuo & Ko 2013).

In both studies done with NLC and SLN has shown to be successful regarding the use of antibodies. EGFRvIII is an epidermal growth factor and is the most common type of EGF receptors present in tumors (Abdolahpour *et al.* 2018). The receptor is suitable as an antibody receiver as the receptor is unusual and almost never identified in healthy cells and tissue (Abdolahpour *et al.* 2018). A anti-EGFRvIII mAb was linked to a hydrophobic drug loaded NLC and the cellular uptake, internalization, was observed. The size of the particle was around 240 nm (Abdolahpour *et al.* 2018) and as mentioned under passive targeting in Section 5.4.2, the maximal size for optimal EPR effect is 200 nm. However, the ligand adaption showed increased internalization in two difference cell lines (Abdolahpour *et al.* 2018).

In another studied carried out by Liu *et al.* (2011) a different antibody was investigated and achieved desirable results (Liu *et al.* 2011). This time the target was a vascular endothelial growth factor receptor (VEGF) a receptor that is present in both tumor neovasculature and in cancer cells (Tol & Punt 2010). The results demonstrated how the human antibody recognized the receptor, VEGFR-2 and inhibited not only the development of new vessels, and thus stifles the supply of oxygen and nutrients to the cancer cells and the TME, but also inhibited tumor growth (Liu *et al.* 2011).

SLN with an antibody ligand have with impressive results manage to cross the BBB and deliver drugs due to the strong affinity the ligand possess for the brain capillaries (Kuo & Ko 2013). The mAb 83-14 was attached to the SLN via a PEG molecule and accordingly to the results, the mAb is a promising antibody for transporting therapeutics to the brain.

5.4.3.5 Ligands are indeed an efficient way to increase specific cellular uptake

Many different kinds of ligands have been used for specific targeting and the results are varied. The choice of ligand needs to be done thoroughly and be adjusted to the purpose. It is obvious that a comprehensive and well-executed research about the target cell needs to be done before choosing ligand. The great amount of research on antibodies are useful but the advantages of Affibody molecules can not be ignored.

5.4.4 The importance of passive targeting for a successful specific cellular uptake and further development of affibodies and antibodies ligands

Lipid based nanoparticles has proved to be easy to modify with a variety of different surface applications. As the EPR effect is not sufficient enough for the level of required concentration for efficient medical treatment or cannot achieve specific targeting to more than evolved cancer states and to the TME, a more advance product is desired. The benefits of being able to use designed LNP for multiple diagnosed diseases was also brought to light and answered the just mentioned aspiration. Even though, today's focus on ligand trials are on the same areas as

the EPR targeting, cancer cells, tumors and TME, there is no doubt that the future will offer more. Antibodies and affibodies can target more than above mention areas and by focusing on surface-specific substances a broader area of diseases can be addressed. Lab engineered affibodies possess the perk of not competing with natural binding material and could therefore be a proper choice when developing ligands to further polish the drug delivery system. To create a safe and functional system it should be of interest to claim resources on identifying unique features for each different pathology. Specific targeting on high loading-capacity drug vehicles, like NLC, can in the long run reduce the total amount of drug administrated to patient and minimize any side effects (Khosa *et al.* 2018).

6 Production

In this section the different production methods of exosomes and lipid nanoparticles will be discussed and evaluated. The analysis will cover the advantages and potential challenges of the particular methods. Specific parameters and other factors that have to be taken into consideration in the manufacturing stage of the production line will be presented.

6.1 Exosome production

Most exosome research is starting with conditioned cell culture media (Gardiner *et al.* 2016), and therefore mainly exosome production starting with cell culture will be discussed in this report. However, a few sentences of exosomes derived from milk are included.

Most of the cultured cells will produce exosomes using standard cell culture media and a standard T-flask or plate. Whitford & Guterstam (2019) states that the challenges of upstream production of exosomes is to continuously produce large quantities of cells without changing cell behavior or characteristics, since such changes could alter the production, composition, attributes or function of the exosomes.

6.1.1 What does a typical exosome production look like?

Small scale culture production typically starts with a culture of adherent cells in a flask or a plate in a standard culture medium (Lamparski *et al.* 2002, Patel *et al.* 2017, Théry *et al.* 2001, Whitford & Guterstam 2019), see Figure 14. The medium is exchanged with a serum-free medium or a medium containing an exosome-depleted serum, after a defined period of time. The change of medium takes place because serum, such as fetal bovine serum (FBS), contain exosomes that can affect the production process (Shelke *et al.* 2014). Many serum-dependent cell cultures will perform relatively normal for a day or two without any serum. The cultures are kept in the new medium for a defined number of hours or days while they secrete exosomes into it. The

medium is then poured out (decanted) and processed for any required vesicle isolation, characterization or purification.

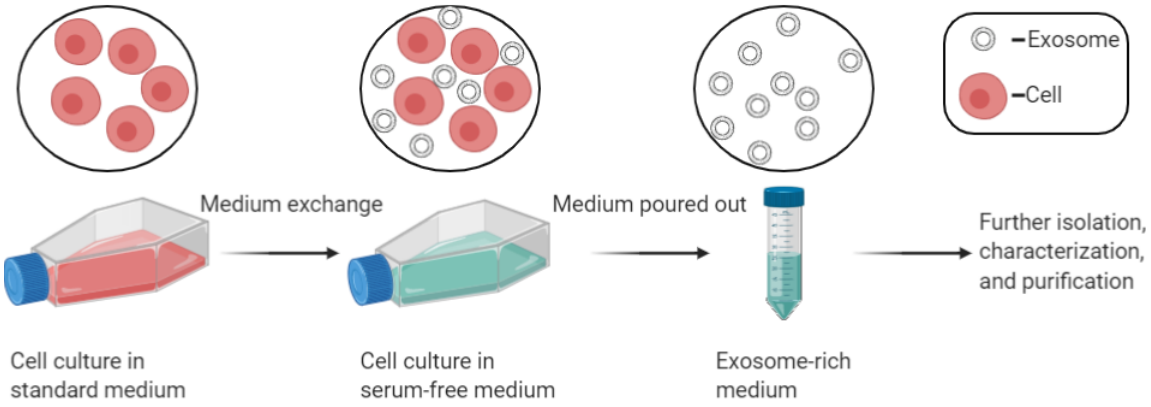

Figure 14. Typical small scale production of exosomes.

6.1.2 Primary and stem cell lines are commonly used to secrete exosomes utilized for drug delivery

Almost all cells produces exosomes but depending on what type of cell it is and in what state it is in, the secreted exosome have different characteristics and content (Whitford & Guterstam 2019). Depending on the application of the exosome it is therefore important to consider which cells to derive the exosome from. For exosomes used for drug delivery mostly primary or stem cell lines have been reported (Cheng *et al.* 2017). Among the companies in the exosome therapeutic market a majority are deriving exosomes from mesenchymal stem cells (MSC), see Table 3 (Aegle-Therapeutics 2020, CellFactory 2020, Kimera-labs 2020, Capricor-Therapeutics 2020, Regeneus 2020, BioRegenerative-Sciences 2020).

Table 3. Companies on the exosome therapeutic market where they explicitly present what type of cells they derive exosomes from (Aegle-Therapeutics 2020, CellFactory 2020, Kimera-labs 2020, Capricor-Therapeutics 2020, Regeneus 2020, BioRegenerative-Sciences 2020).

Companies on the exosome diagnostic market	Cell type
Aegle Therapeutics	Bone marrow - MSC
BioRegenerative Sciences	Human Adipose Derived Stem Cell
Capricor Therapeutics	Cardiosphere-derived cells
Cell Factory	MSC
Kimera Labs	MSC
Regeneus	Adipose-derived - MSC

Another interesting source being reported for exosome production is bovine milk (Munagala *et al.* 2016). It is a cost-effective alternative for bulk quantities. A study on mice have shown that oral intake of milk-derived exosomes loaded with PAC have significantly higher therapeutic efficacy and reduced tissue toxicities compared to PAC i.v. treatment (Agrawal *et al.* 2017).

6.1.3 A great amount of factors can impact the exosome production

A big challenge with exosome production is to produce exosomes with constant characteristics. The challenge arises because there are a lot of factors that can affect the production of exosomes, this includes the characteristics of the source, how the handling of the source material is done, and the experimental conditions (Théry *et al.* 2018). For example can the passage number, the number of times a cell line has been subcultured, affect exosomes vascularization bioactivity (Patel *et al.* 2017). A decrease in cell seeding density can also increase the exosomes produced per cell. Furthermore can an increased frequency of exosome collection result in a higher yield of exosomes. Wang *et al.* (2014) shows that the oxygen concentration in cell culture also can be a factor. A low concentration of oxygen can increase the number of exosomes produced per cell (Umezu *et al.* 2014, Wang *et al.* 2014). Supplements in the culture medium impacts the exosome production as well. Rice *et al.* (2015) reports that the glucose concentration can affect both the bioactivity and the rate of release of exosomes. In another study, Németh *et al.* (2017) found that ciprofloxacin, an antibiotic, in the culture medium induced the release of exosome associated DNA. Consequently it shows that antibiotics can have an effect on the molecular composition of exosomes. Another supplement that can cause an effect on exosome secretion is growth factors (Zhou *et al.* 2017). To summarize, there are a lot of factors that can affect the output of exosome production and it is important to consider them all to keep the exosomes characteristics constant.

6.1.4 Advantages and disadvantages with serum, exosome-depleted serum and serum-free media

Medium components with the greatest impact on exosome production are serum and other complex components that are likely to contain exosomes (Pachler *et al.* 2017, Shelke *et al.* 2014). The exosomes from the serum are hard to distinguish from the cell-derived exosomes, which complicates the purification of cell derived-exosomes. Shelke *et al.* (2014) also showed that FBS extracellular vesicles (EVs) have a direct migratory effect on epithelial cancer cell line, meaning that the serum-derived EVs can affect the cell. They further present a protocol to deplete the EVs from FBS through Ultracentrifugation for 18 hours. However, Eitan *et al.* (2015) showed that EV-depleted FBS reduces the growth rate of cells and Beninson & Fleshner (2015) reports that culturing cells in FBS depleted of exosomes can significantly alter the immunological activity of the cells. This indicates that exosome-depleted FBS may give a lower yield and change cell behavior which suggests that it is not a great option. Furthermore, Li *et al.* (2015) did a comparison of N2a neuroblastoma cells cultured in EV-depleted serum and serum-free media. They concluded that the quantity of EVs greatly increased in the serum-free media but the composition of the EVs could alter. Serum-free media could be an option but the risk of change in exosome composition is negative. In conclusion is the choice of media and particularly the choice of serum, EV-depleted

serum or serum-free media something to consider for the production of exosomes and something that needs more research.

6.1.5 Mycoplasma is a contaminant

Contamination from Mycoplasma can occur during the production, because it can both produce EVs (Chernov *et al.* 2014) and change characteristics of the cell (Yang *et al.* 2012, Corral-Vázquez *et al.* 2017). The EVs produced can overlap in size with the cell-derived exosomes and have similar characteristics which complicates the purification. Furthermore can the altered cell characteristics lead to changed exosome composition. Therefore should regular checks for such contaminations be done during the manufacture. This is also a reason to culture cells in an aseptic milieu and to strive for as many closed systems as possible.

6.1.6 Large-scale production of exosomes

The potential methods to scale up the production of exosomes are the use of many large T-flasks, several multilayered culture flasks, stirred tank bioreactor employing microcarriers, and the most prominent method perfusion-based hollow-fiber bioreactor (Colao *et al.* 2018). The overall challenges with large-scale, and clinically relevant, manufacture of exosomes includes steps such as process optimization, confirmation, validation and characterization, and process performance qualification activities (Whitford & Guterstam 2019). Furthermore are there new and higher industry goals, such as heightened process control, establishing as many closed operations as possible, moving to more automated and digital processes, and using single-use systems.

6.1.6.1 Many large T-flasks

One potential method to scale up the production of exosomes is to do what is done small-scale and just increase the number of flasks (Whitford & Guterstam 2019). Since most cells cultured for exosome production are adherent cells, the T-flasks are coated with an extracellular matrix on its inner surface. The extracellular matrix is causing the cells to adhere to the surface and makes it possible for the cells to proliferate. The already gathered knowledge manufacturing with T-flasks is an advantage and the time consuming step of learning a new method is avoided. Disadvantages with this method is the cost of culture expansion before the actual production phase, it gives relatively low yield so lots of flasks are needed and it can take time to establish a reproducible culture that can be verified (Haraszti *et al.* 2018, Yan & Wu 2019).

6.1.6.2 Several multilayered culture flasks

This method is practically the same as many large T-flasks, except that every flask have a few layers. The addition of layers results in a greater surface area, which makes it possible to grow more cells. The advantage of this method is that it does not need as many flasks and therefore does not take as much space as the conventional culture flasks. However, the challenges are the same as for the large T-flasks and it is more expensive than standard T-flasks (Whitford & Guterstam 2019).

6.1.6.3 Stirred tank bioreactors employing microcarriers

This method utilizes a bioreactor with an impeller that continuously stirs the culture medium so the nutrition and growth factors distributes evenly in the reactor (Phan *et al.* 2018). Microcarriers are small microscopic beads that anchorage-dependent cells can grow on (Chen *et al.* 2011). It can be used in impeller-based bioreactors to increase the surface where the adherent cells can proliferate. Advantages with this method are that it produces a relative high yield of exosomes (Haraszti *et al.* 2018). However, there are challenges with the efficiency of cell-to-microcarrier bindings and some cells are extremely sensitive to the hydrodynamic forces caused by the impeller, which can alter the phenotype of the cell and consequently change the exosome composition (Chen *et al.* 2011).

6.1.6.4 Hollow-fiber bioreactor

This method is a three-dimensional culture system that utilizes small semipermeable hollow fibers that are contained in a cartridge (Yan *et al.* 2018). The inside of the cartridge is divided into the intracapillary space (ICS), which is the inside of the fibers and the extracapillary space (ECS). Cells are cultured either in the ICS or the ECS and culture media is continuously pumped through the fibers on the opposite side of the membrane (Whitford *et al.* 2015). The fibers have a defined molecular weight cutoff (MWCO) around 5-20 kDa which allows nutrients, gases and waste products to pass through but prevents exosomes and cells to go through, see Figure 15.

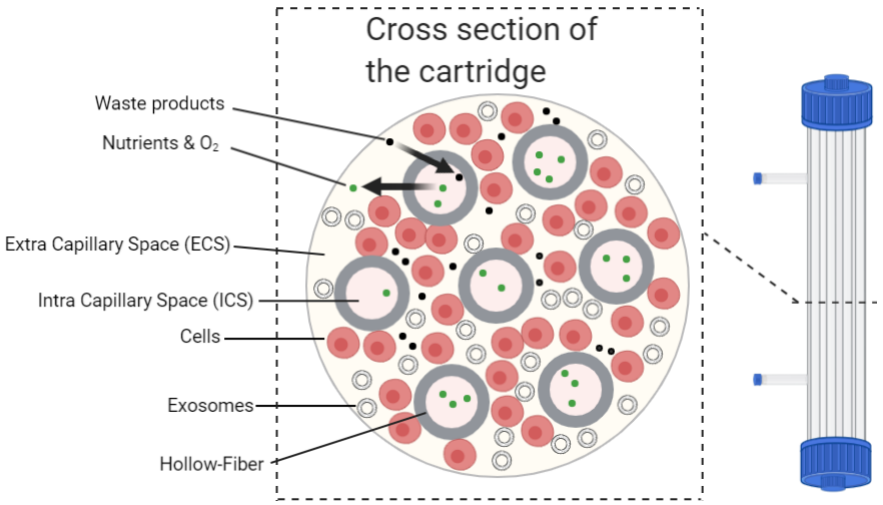

Figure 15. The schematics of a Hollow-Fiber Bioreactor and its cross section. The arrows show how waste products, nutrients and gases permeate through the membrane of the hollow fibers. It enables an environment where the cells can proliferate and continuously produce exosomes.

This method generates a high yield (Watson *et al.* 2016, Yan & Wu 2019), it has the advantage of reduced risk of contamination being a closed system and finally, the flow of media can be automated leading to less labor. Whitford *et al.* (2015) showed that human adipose-derived MSC could be maintained in a hollow-fiber reactor and continuously produce exosomes for 10 weeks, without splitting and subculturing the cells. This indicates that

exosomes can be manufactured for a long time using this method. A disadvantage to this method is that it is relatively new and it can therefore take time to optimize all parameters and ensure that the exosomes produced have a consistent composition.

6.1.7 Which exosome production method is most optimal

One of the big challenges for exosome therapy to reach the clinic, is to produce a sufficiently large amount of exosomes with constant characteristics (Colao *et al.* 2018). To overcome this challenge a scale up of the production is needed. Comparing the aforementioned potential methods for large-scale production, Hollow-Fiber Bioreactor shows most potential. Although it has not been used broadly, it gives higher yield than the conventionally used T-flasks and it avoids impeller-based hydrodynamic forces that the Stirred-Tank Bioreactor generates (Watson *et al.* 2016, Chen *et al.* 2011). Furthermore can it alleviate some concerns of the higher industry goals, having potential to be an automated and closed system. The ability to continuously produce exosomes for a long period of time is a major advantage for the Hollow-Fiber Bioreactor, as subculturing can have an effect on the characteristics of the exosome and it requires labor. However, more research needs to be done to optimize all the parameters affecting the exosome production and to validate that the exosomes have constant characteristics.

6.2 The production of lipid nanoparticles for drug delivery

The focal point of this section is the synthetic production of lipid nanoparticles and the potential hurdles of the established production methods.

6.2.1 The choice of production method highly influences the characteristics of the lipid nanoparticles

The performance and main characteristics of LNP are highly influenced by the selection of production method (Shah *et al.* 2015). In this section, different approaches for the synthesis of LNP will be evaluated and potential hurdles including scalability and drug stability issues will be discussed. The selection of method is further affected by energy requirements, degree of hazard, feasibility and yield (Ganesan & Narayanasamy 2017). Moreover, the physicochemical properties of the drug, stability of the drug, stability of the formed LNP and the availability of equipment have to be taken into consideration (Shah *et al.* 2015). A polydispersity index is commonly used to describe the molecular weight distribution and is applicable for lipid nanoparticles (Rogošić *et al.* 1996). The characteristics of melting, control of release kinetics and drug encapsulation efficiency are affected by the polydispersity of the LNP (Rawat *et al.* 2008). Therefore, the required degree of polydispersity must be taken into consideration when choosing the most suitable production method.

LNP production methods are frequently categorized into two groups; high energy approaches for the dispersion

of the lipid phase and precipitation techniques (Shah *et al.* 2015). The most commonly used methods for formation of LNP in the first category include High-Pressure Homogenization and Ultrasonication. Microemulsion, Solvent Evaporation and Supercritical Fluid Extraction and Microfluidic devices are associated with the second category (Shah *et al.* 2015). The mechanisms of these methods along with their identified advantages and disadvantages will be discussed in this section.

High-Pressure Homogenization (HPH) is a well-established method to produce LNP (Yoon *et al.* 2013). However, the exposure of the encapsulated drugs to high temperatures and pressures has led to development of alternative methods. HPH and Microemulsion are the two most used methods to produce LNPs and their abilities to scale-up has been frequently discussed (Wissing *et al.* 2004). Some of the established methods use organic solvent that can cause toxicological issues (Yoon *et al.* 2013). Despite the environmental concerns of organic solvents, the methods have their advantages, the largest one being the mild operating conditions that can easily handle thermo-sensitive drugs (Yoon *et al.* 2013). The ability to scale up the production of LNPs is important when it comes to getting a product out on the market.

6.2.2 High-Pressure Homogenization - the most established technique for production of lipid nanoparticles

High-Pressure Homogenization (HPH) as the most influential and reliable method to produce LNP (Shah *et al.* 2015). HPH is a well-established method and have been used the last decades for large-scale production of solid lipid nanoparticles (SLN) (Yoon *et al.* 2013). In 1996 HPH was patented for the production of SLN by Müller and Lucks, however the method was introduced beforehand by Siekmann and Westesen in 1992 (Shah *et al.* 2015). HPH can be performed using two different approaches; at high temperatures or below room temperature, using Hot HPH technique and Cold HPH technique respectively (Wissing *et al.* 2004). The size of the particles is controlled by changing cavitation and turbulences and both techniques can generate narrow particle distributions with a polydispersity index less than 0,2 (Lippacher *et al.* 2001). As Ganesan & Narayanasamy (2017) point out, they both have in common that the dispersing technique is very effective. Molecules with high molecular weight and long chains, like RNA and DNA are especially sensitive to HPH and drugs consisting of these particular molecules should be produced using a different approach. (Rawat *et al.* 2008). The advantages and disadvantages of the Hot and Cold HPH approaches will be discussed in Section 6.2.2.1 and 6.2.2.2.

6.2.2.1 Hot High-Pressure Homogenization effectively disperses lipid nanoparticles

The Hot HPH is an energy intensive approach where the LNP are prepared at temperatures above the melting point of the solid lipids. Initially, the temperature is elevated to around 5-10°C above the melting point of the lipid (Kovačević *et al.* 2020). The lipids are melted, and subsequent solubilisation or dispersion of the drug is then followed by an additional step of integration with an aqueous surfactant solution prepared at the same temperature by high speed stirring (Shah *et al.* 2015). The dispersion forms a hot pre-emulsion, which is further processed in a high-pressure homogenizer (Shah *et al.* 2015, Kovačević *et al.* 2020). The temperature is kept at 5-10°C to prevent recrystallization of the lipids and intense cavitation is generated by the large pressure drop

through the homogenizer (Kovačević *et al.* 2020). The number of cycles is generally no more than five at 500 bar (Lippacher *et al.* 2001, Salvi & Pawar 2019, Kovačević *et al.* 2020). The number of cycles can be altered depending on the desired size of the LNP (Salvi & Pawar 2019).

SLN are developed upon cooling down to room temperature which induces recrystallization of the solid lipids in the hot colloidal emulsion (Lippacher *et al.* 2001). A water bath can be used for controlled cooling and regulation of the velocity of recrystallization (Kovačević *et al.* 2020). There have been occasional cases where lower temperature conditions, or even sub-zero temperatures have been beneficial (Unruh *et al.* 2001, Lim & Kim 2002). Figure 16 gives a schematic illustration of the steps in the Hot HPH technique.

Beside the composition of the dispersion, the size of LNP depends on the homogenization parameters (Dingler & Gohla 2002). A modification of the homogenization time, an increase of the homogenization temperature and an adjustment of the pressure can decrease the size of the formed particles (Dingler & Gohla 2002, Jenning *et al.* 2002, Yang & Zhu 2002). The typical particle size in Hot HPH range from 70 to 270 nm (Dingler & Gohla 2002, Jenning *et al.* 2002, Shi *et al.* 2012, Desai & Thakkar 2016).

The factors that make Hot HPH a suitable selection are the easily scalable feature, reproduciblity and the fact that homogenizers are commercially available (Ganesan & Narayanasamy 2017). In addition, the method is well-established and effectively disperses LNP in the high amount of lipids. The method is limited to hydrophobic drugs since hydrophilic drugs are inappropriate in this technique due to their distribution in the aqueous phase (Shah *et al.* 2015).The LNP are exposed to high temperatures and shear by intense turbulent eddies which may expose the encapsulated drugs to thermodynamic and mechanical stress (Yoon *et al.* 2013). Therefore, Hot HPH is not applicable for thermolabile drugs since complete evasion of exposure to high temperatures is impossible. Modifications and super-cooled melts can be formed during crystallization of the nanoemulsion (Ganesan & Narayanasamy 2017). The usage of heat and shear forces requires intensely high energy inputs (Shah *et al.* 2015). The formed LNP are highly polydisperse and the elevated temperatures in the process leads to a reduction in efficiency of homogenization (Shah *et al.* 2015). In the production of LNP encapsulated with thermo-labile drugs, Cold HPH is a valuable alternative to Hot HPH since it avoids thermo-induced drug degradation.

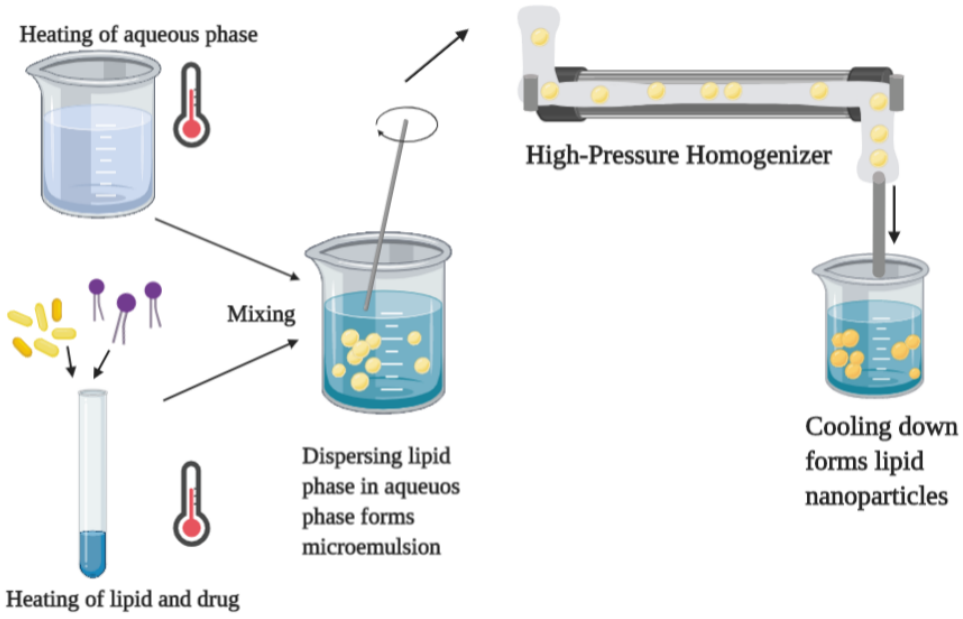

Figure 16. Schematic illustration of the steps in the Hot High-Pressure Homogenization method used to produce drug-loaded lipid nanoparticles. The lipid phase with the drug and the aqueous phase is heated separately to the same temperature. Mixing of the two phases under stirring forms a hot Microemulsion and subsequent Hot High-Pressure Homogenization leads to a colloidal emuslion which precipitates to lipid nanoparticles after cooling down.

6.2.2.2 Cold High-Pressure Homogenization can reduce thermo-induced drug degradation

Temperature labile or hydrophilic drugs processed in HPH are generally performed using the cold technique (Wissing *et al.* 2004). Following the melting of lipids and drug, the exposure to liquid nitrogen or dry ice solidifies the drug-containing lipid melt (Lim & Kim 2002, Wissing *et al.* 2004). The distribution of the drug in the lipid phase is favoured by rapid cooling (Shah *et al.* 2015). Microparticles are formed by milling of the solid phase into a fine powder (Lim & Kim 2002, Kasongo *et al.* 2012). High speed stirring of the microparticles in a cold aqueous surfactant solution forms a pre-suspension (Wissing *et al.* 2004). SLN are generated when the pre-suspension is High-Pressure Homogenized at temperatures below the melting point of the lipid (Kasongo *et al.* 2012). The conditions for homogenization are generally no more than five cycles at 500 bar (Wissing *et al.* 2004, Kasongo *et al.* 2012, Salvi & Pawar 2019, Kovačević *et al.* 2020). Figure 17 gives a schematic overview of the Cold HPH technique.

Cold HPH effectively disperses the particles and is highly suitable for thermo-sensitive drugs (Shah *et al.* 2015). The method have the ability to scale-up and is applicable for hydrophilic drugs since the drug loss is reduced by the shorten of lipid melting (Shah *et al.* 2015). The advantages of Cold HPH over Hot HPH involves no drug degradation or crystalline modifications of LNP due to increased temperatures (Wissing *et al.* 2004). In addition, the drug-loading capacity is increased in Cold HPH by rapid cooling. Cold HPH involves more harsh conditions than Hot HPH due to the fact that the dispersion of solid lipids requires higher energy inputs (Shah *et al.* 2015). As a result, Hot HPH is more effective and smaller as well as more monodisperse particles are generated (Shah *et al.* 2015). An additional disadvantage of Cold HPH is the risk of drug dischargement during storage of the formed LNP.

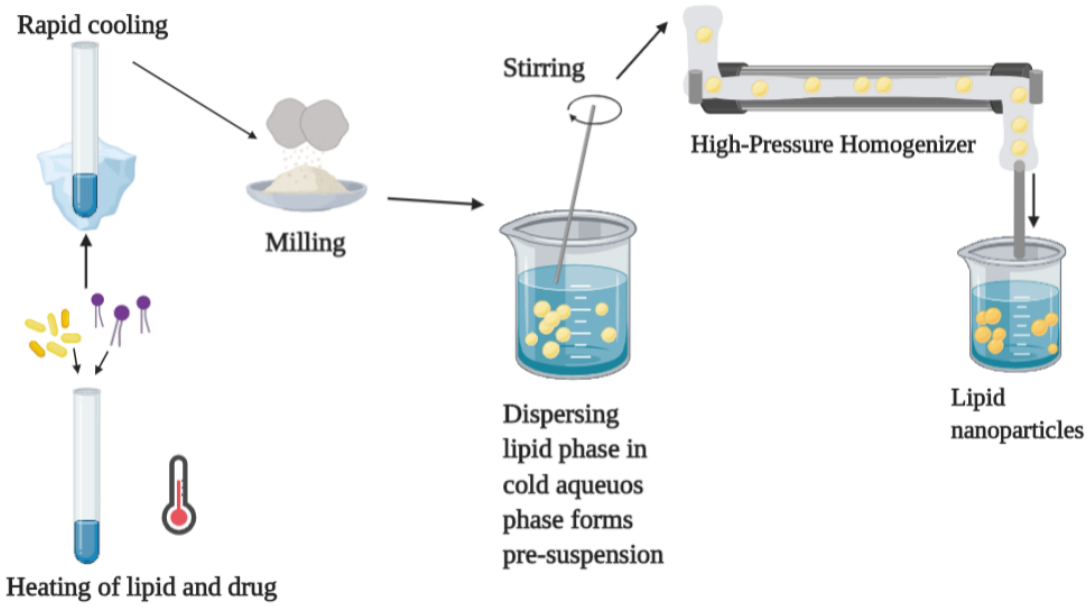

Figure 17. Schematic overview of the steps in the Cold High-Pressure Homogenization method used to produce drug-loaded lipid nanoparticles. The lipid phase with the drug is heated and rapidly cooled. Milling and dispersion under stirring forms a pre-suspension. The Cold High-Pressure Homogenizer forms lipid nanoparticles.

6.2.3 Ultrasonication forms particles with small sizes

In the Ultrasonication process, cavitation causes formation and fricative collapse of globules in a liquid (Ganesan & Narayanasamy 2017). The cavitation is created by ultrasound waves and the collapse of globules lead to an site-specific increase in temperature and pressure (Cheaburu-Yilmaz *et al.* 2019). Initially, the drug and lipids are collectively melted at 5-10°C (same temperature as in Hot HPH, see Section 6.2.2.1) above the melting point of the solid lipids in the mixture (Salvi & Pawar 2019). Additionally, an aqueous phase, usually distilled water with surfactants is heated to the same temperature (El-Helw & Fahmy 2015). The different phases are mixed and subjected to high shearing (Salvi & Pawar 2019). Subsequently, the emulsion is ultrasonicated, mixed with a specific amount of distilled water and lastly cooled down to promote formation of LNP (El-Helw & Fahmy 2015). Figure 18 presents an overview of the Ultrasonication procedure.

The small size of the formed particles is an advantage of Ultrasonication and range from 30-180 nm, as compared to 70-270 nm in Hot HPH (Ganesan & Narayanasamy 2017). Furthermore, Ultrasonication cause low shear stress (deformation forces) on the LNP (Ganesan & Narayanasamy 2017). However, the process involves contamination due to metal shading and has a relatively low entrapment efficiency (Ganesan & Narayanasamy 2017). Ultrasonication is an energy intensive process and scalability of the production has not been tested (Ganesan & Narayanasamy 2017). The scalability issue has to be ressolved before Ultrasonication can be established as a standardized procedure for production of LNP in the area of drug delivery.

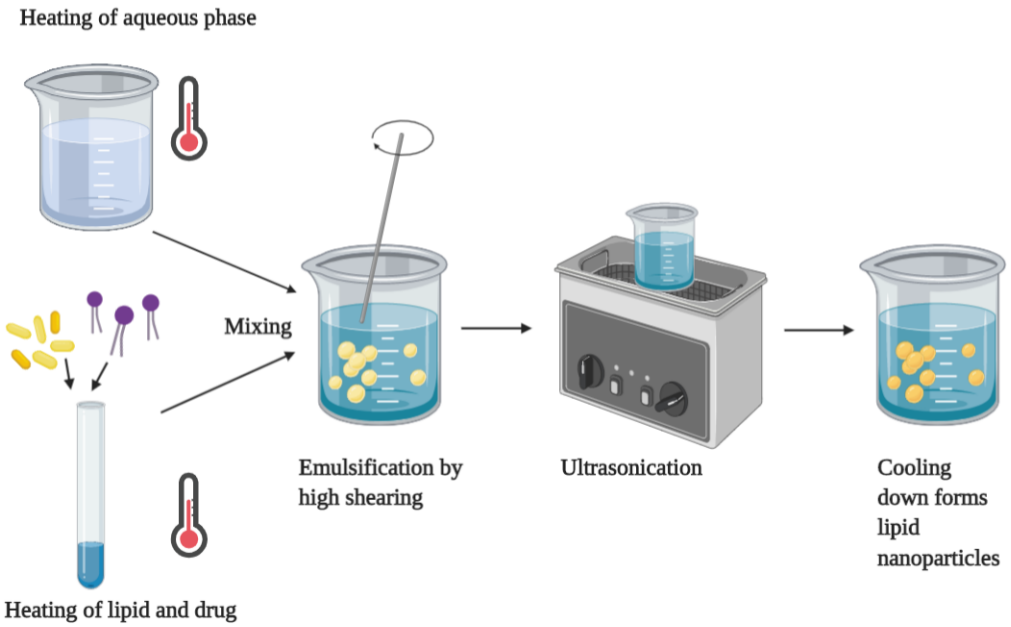

Figure 18. Schematic overview of the Ultrasonication method used to produce drug-loaded lipid nanoparticles. The lipid phase with the drug and the aqueous phase is heated separately to the same temperature. Ultrasonication of the mixture creates an emulsion and subsequent cooling down leads to lipid nanoparticle formation.

6.2.4 Microemulsion disperses lipid nanoparticles with high stability

Emulsification is the most common production technique of LNP and the production of SLN by precipitation from hot microemulsions was described and patented in 1993 by Gasco (Shah *et al.* 2015, Saghazadeh *et al.* 2018, Gasco 1993). LNP are formed due to a reduction in spontaneous interfacial tension (Ganesan & Narayanasamy 2017). The mixing of two immiscible phases forms colloidal particles of lipids (Gasco 1993, Jiang *et al.* 2013). The lipid phase is heated to a temperature above its melting point and the drug is solubilised in the melted lipid (Gasco 1993). The aqueous phase, including a surfactant and occasional co-surfactant is heated separately from the lipid phase to the same temperature (Gasco 1993, Joshi & Patravale 2006). The lipid melt is added to the aqueous phase and continuous mechanical stirring forms a hot clear microemulsion (Gasco 1993). The rapid dispersion of the hot microemulsion in cold water, typically in 2-10°C, forms LNP by lipid crystallization (Gasco 1993, Joshi & Patravale 2006). In general, the ratios of the lipid melt:aqueous phase are 1:25 or 1:50 (Gasco 1993). An overview of the steps in the Microemulsion process is presented in Figure 19.

In Microemulsion, there is no need for high energy input and the formed LNP are theoretically stable (Ganesan & Narayanasamy 2017). The method is easy to scale-up and sophisticated equipment is not required (Shah *et al.* 2015). Particle aggregation can be avoided by fast crystallization of lipids due to high temperature alterations (Shah *et al.* 2015). The alternation of mixing speed and viscosity of the solution facilitates the control of the particle size (Saghazadeh *et al.* 2018). Even though the method has issues with polydispersity, the formed particle are usually spherical which is an advantages regarding the release profiles of the LNP (Saghazadeh *et al.* 2018).

The process is highly sensitive to change and requires labor intensive work (Ganesan & Narayanasamy 2017). The concentration of the formed LNP is relatively low due to the large amount of water required for formation by dilution (Ganesan & Narayanasamy 2017). Large volume of water used in the process creates a strong dilution of LNP which entails low lipid content.

LNP can also be prepared using a double Microemulsion technique that generates a water-in-oil-in-water emulsion by an additional emulsification step (Yoon *et al.* 2013, Saghazadeh *et al.* 2018). Cold water, typically 2-10°C is used to precipitate LNP with a smaller size and more narrow size range, by dilution of the double microemulsion (Jiang *et al.* 2013, Yoon *et al.* 2013).

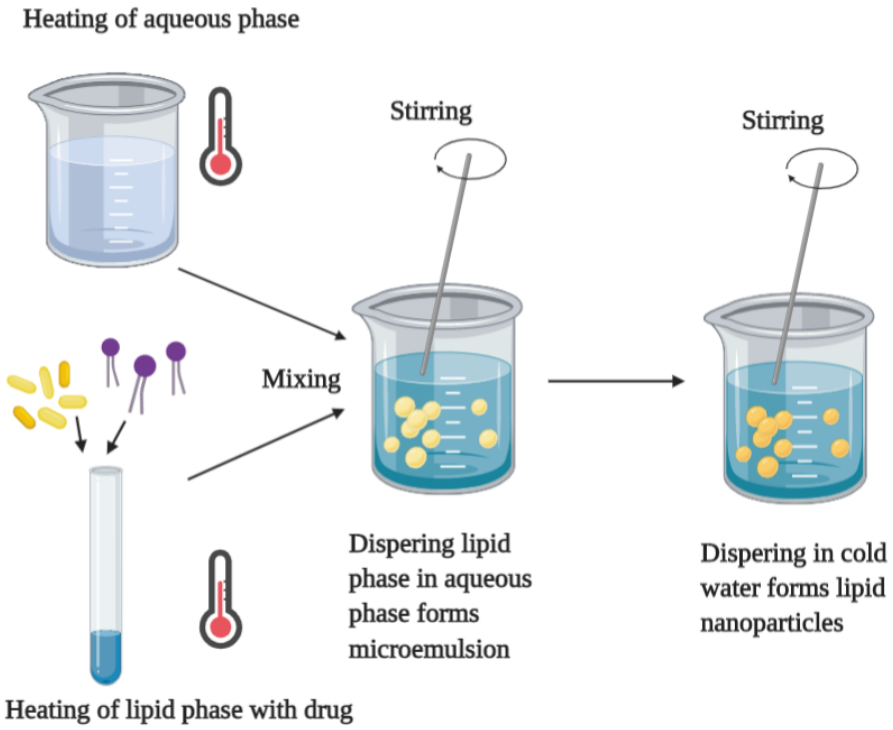

Figure 19. Schematic overview of the Microemulsion method used to produce drug-loaded lipid nanoparticles. The lipid phase with the drug and the aqueous phase is heated separately to the same temperature. Mixing of the two phases under stirring forms a hot Microemulsion and subsequent dispersing in cold water leads to lipid nanoparticle formation.

6.2.5 Solvent Evaporation - applicable for thermo-labile drugs

In the Solvent Evaporation method, organic solvents like cyclohexane, chloroform or ethyl acetate are used to dissolve the lipids along with lipophilic drugs (Cortesi *et al.* 2002, Shah *et al.* 2015). An aqueous anti-solvent phase of surfactant is used to emulsify the organic phase (Cortesi *et al.* 2002). Precipitation during complete evaporation of the organic solvent under reduced pressure forms LNP (Cortesi *et al.* 2002). A schematic overview of the Solvent Evaporation method can be seen in Figure 20.

The process generates LNP with a low size range and evades thermolabile drug degradation by no increased temperatures (Ganesan & Narayanasamy 2017). Solvent Evaporation does not require high energy input and the

formed system has a low viscosity (Ganesan & Narayanasamy 2017). There is also no demand of a highly dilute LNP dispersion (Ganesan & Narayanasamy 2017). Moreover, the method does not involve sophisticated equipment and is easy to scale-up (Shah *et al.* 2015).

However, Solvent Evaporation has problems with low dispersing degree and emulsion instability (Ganesan & Narayanasamy 2017). The organic solvents aggravates the solubility of lipids and residual solvent present in the final stage can cause toxicological issues (Ganesan & Narayanasamy 2017). There can be risks of particles aggregation if the evaporation is not fast enough (Shah *et al.* 2015). The advantages including low size range and avoidance of drug degradation have to be balanced against the consequences of the use of organic solvents.

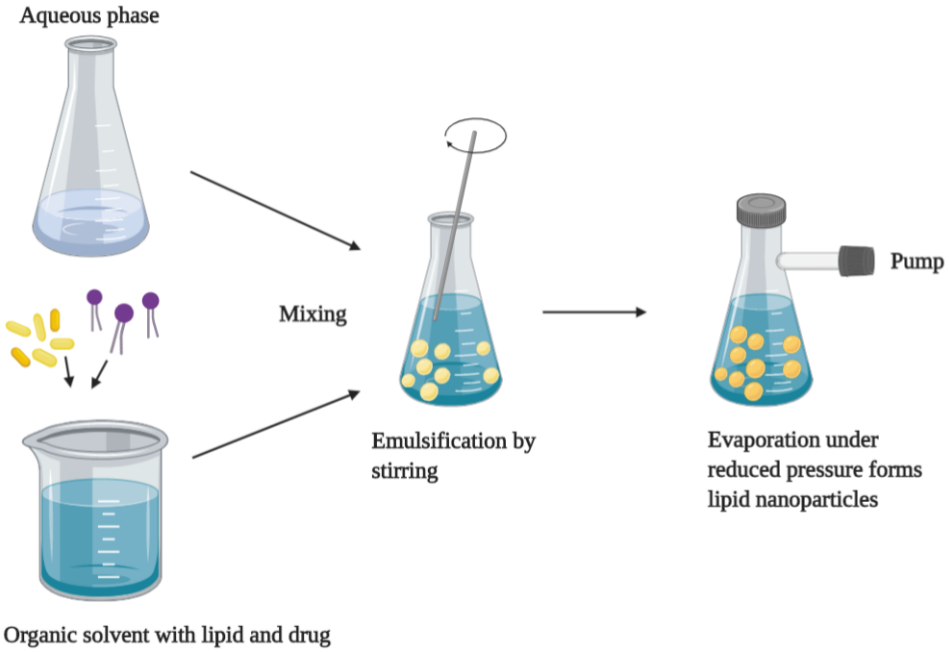

Figure 20. Illustration of the steps in the Solvent Evaporation method used to produce drug-loaded lipid nanoparticles. The drug and lipid is dissolved in the organic solvent. Subsequent mixing of the two phases creates an emulsion. The organic solvent is evaporated under reduced pressure and LNP are formed.

6.2.6 Supercritical Fluid Extraction method forms monodisperse particles

The Supercritical Extraction method use organic solvents to produce monodisperse lipid nanoparticles (Shah *et al.* 2015). Firstly, the lipid and drug are dissolved and heated in an organic solvent such as chloroform and a suitable surfactant is added (Chattopadhyay *et al.* 2007, Shah *et al.* 2015). Secondly, the formed organic solution is added to a pre-heated aqueous solution with an optional co-surfactant and subsequent HPH forms a colloidal emulsion (Chattopadhyay *et al.* 2007).

A good candidate for a supercritical fluid that acts as an anti-solvent is a carbon dioxide solution (Chattopadhyay *et al.* 2007). The complete or partial miscibility of the supercritical fluid to the solvent allows for precipitation of the lipids (Shah *et al.* 2015). The supercritical fluid is prepared by maintaining a constant temperature and pressure (Chattopadhyay *et al.* 2007). The emulsion is sprayed at a constant rate onto one end of an extraction column and the supercritical fluid is added from the other end at a constant flow rate (Chattopadhyay *et al.* 2007,

Shah *et al.* 2015). If the solution is not in gas form, the mixture is atomized beforehand through the nozzle of the column (Joshi & Patravale 2006). Multiple Supercritical Fluid Extractions of organic solvent from the emulsion and dissolved lipids are performed in parallel (Chattopadhyay *et al.* 2007, Ganesan & Narayanasamy 2017). The lipids are crystallized and the continuous extraction of the organic solvent contributes to LNP formation (Chattopadhyay *et al.* 2007). Figure 21 presents an outline of the different steps in the Supercritical Fluid Extraction process.

The final LNP are collected as dry powder (Ganesan & Narayanasamy 2017). The low temperature and pressure prevents drug degradation (Ganesan & Narayanasamy 2017). Solvent removal is rapid and efficient and the formed LNP are highly monodisperse (Shah *et al.* 2015). The use of supercritical fluids facilitates the removal of impurities such as low molecular weight compounds (Shah *et al.* 2015). The melting point of lipids is reduced by supercritical fluids which means the method is suitable for thermo-labile drugs (Shah *et al.* 2015). An additional advantage of the method is the thermodynamic stability of the LNP dispersion caused by the decrease of viscosity of the liquid due to carbon dioxide characteristics as a supercritical fluid (Shah *et al.* 2015).

The disadvantages of using supercritical fluids include that the method is expensive and the process requires the use of organic solvents (Shah *et al.* 2015, Ganesan & Narayanasamy 2017). In some cases, complete control of drug release profile is required and monodisperse particles is therefore a necessity. The Supercritical Extraction method has great potential in producing lipid nanoparticles with predictable release profile but the use of organic solvents is an obstacle that has to be tackled before the technique can progress into the front line of lipid nanoparticle production methods.

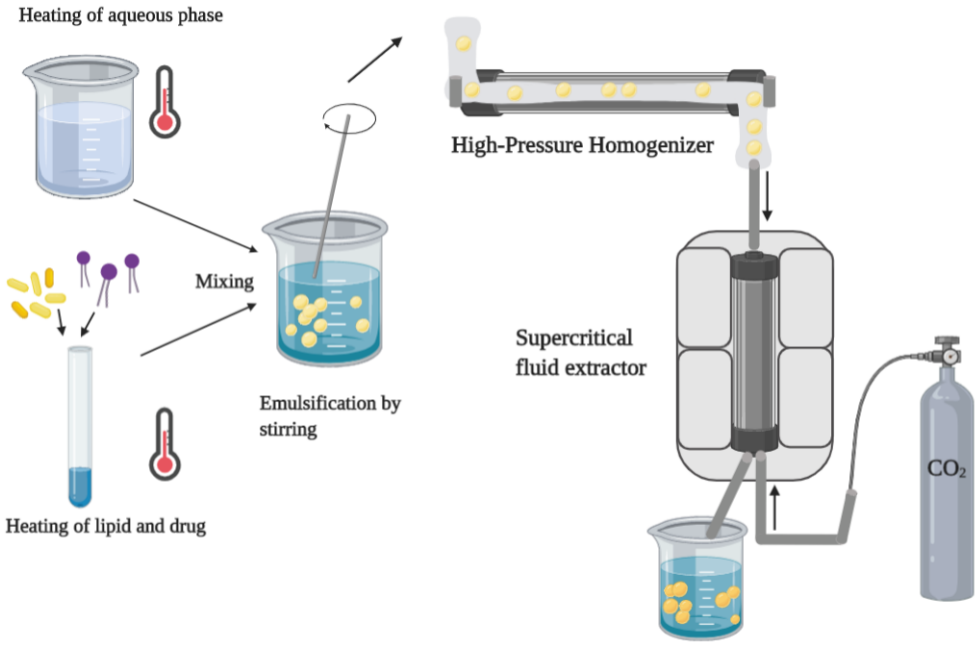

Figure 21. Outline of the Supercritical Fluid Extraction process that produce drug-loaded lipid nanoparticles using a high-pressure homogenizer and a supercritical fluid extractor. The lipid and drug is heated and dissolved in an organic solvent. The mixing of the organic phase with a pre-heated aqueous phase forms an emulsion. High-Pressure Homogenization and subsequent Supercritical Fluid Extraction forms lipid nanoparticles.

6.2.7 Microfluidics platforms - a rapidly developing field

Microfluidic devices have successfully been used as tools in the manufacturing of LNP (Riewe *et al.* 2020). An advantage of microfluidics is the ability to manipulate the flow rate which enables a consistent mixing ratio of the components (Streck *et al.* 2019). In this section, two different approaches will be presented and optimization of the device parameters will be discussed.

6.2.7.1 Anti-solvent approach - most commonly used

The production of LNP on microfluidic platforms are primarily based on an anti-solvent approach (Streck *et al.* 2019). The anti-solvent approach was used by Riewe *et al.* (2020) on a microfluidic device where lipids dissolved in ethanol were combined with an aqueous stream. In conclusion, a co-solvent system where the solubility of the lipids is lower than in the solvent only, is formed after mixing of the streams in the anti-solvent technique. LNP formation by precipitation of the lipids is promoted by the decrease in solubility and subsequent removal of ethanol by evaporation (Streck *et al.* 2019). An overview of the setup of a microfluidic device using the anti-solvent approach can be seen in Figure 22.

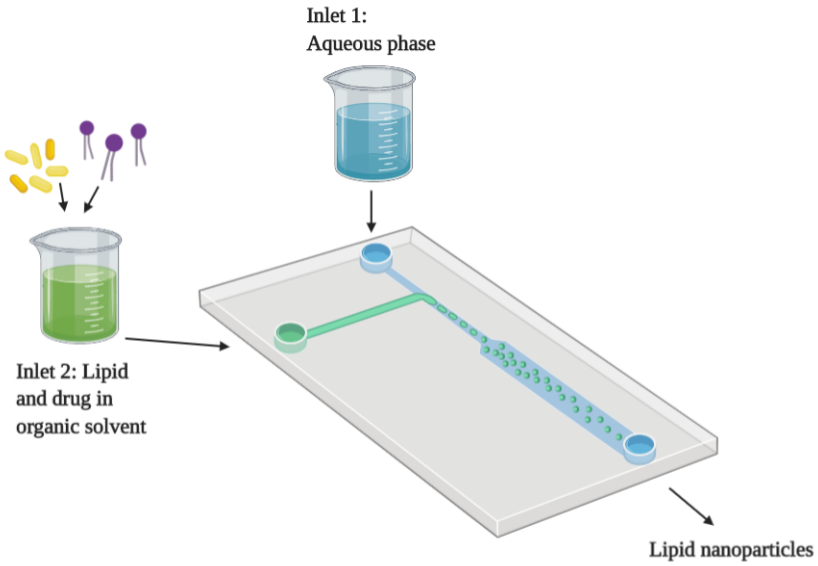

Figure 22. Overview of a microfluidic device that produce drug-loaded lipid nanoparticles using the anti-solvent approach. A lipid-solouble drug is dissolved in lipids with an organic solvent and enters the microfluidic platform through inlet 2. The organic phase is mixed with the aqueous phase from inlet 1 in the unified channel and forms lipid nanoparticles that leave the device through the outlet.

6.2.7.2 Droplet-based channel designs avoid organic solvents

The need of subsequent removal of residual organic solvent can be avoided using microfluidic devices with droplet-based channel designs (Streck *et al.* 2019). Even though the formed emulsions can encapsulate drugs, the majority of research of droplet-based channel designs has been focused on food applications as opposed to drug delivery (Streck *et al.* 2019).

6.2.7.3 Optimization - microfluidic parameters influence the characteristics of the particles

Parameters like sample concentration and flow rate ratio of the microfluidic device can be varied to control the particle size distribution (Karnik *et al.* 2008). The flow rate ratio in a microfluidic device is defined as the ratio between the anti-solvent or aqueous phase stream and the organic solvent stream (Streck *et al.* 2019). A narrow size range can be achieved by increasing the flow rate ratio in the formation of LNP (Kastner *et al.* 2014).

Flow rate is defined as the combined speed of the different solvents in a microchannel (Streck *et al.* 2019). The size of LNP was not affected by a modification of the flow rate (Kastner *et al.* 2014). However, at flow rates above 15 µL/min the lipid nanoparticles were exposed to shear stresses (Ghazal *et al.* 2017). As a result, the orientation of the lipids in the crystallization was aligned with the direction of the flow (Ghazal *et al.* 2017). Consequently, a modification of the flow rate and thus equilibrium state due to shear stress affect the structure of the LNP (Ghazal *et al.* 2017, Streck *et al.* 2019).

Microfluidic devices are applicable for expensive and low volume samples but has yet to be developed for large-scale production (Riewe *et al.* 2020). The continuous flow rates ensure reliable quality of the formed LNP (Riewe *et al.* 2020). The run-time for a low volume system is very long, however, the possibility of parallel systems is a prominent scaling strategy (Riewe *et al.* 2020). Anderluzzi *et al.* (2019) have presented a method that they estimate being able to scale from bench to clinic. Further, they were also able to reduce particle size, polydispersity and alternate parameters, e.g. pressure.

6.2.7.4 Drug entrapment in upcoming areas

The hydrophilic drugs are dissolved in the aqueous solvent and the hydrophobic drugs are dissolved in the lipid phase (Streck *et al.* 2019). Microfluidic devices are highly suitable for the production of RNA incorporated in LNP and increase drug release by efficient encapsulation (Veiga *et al.* 2020). There are several ongoing clinical trials in the manufacture of RNA-loaded LNP and they have shown therapeutic potential in areas cancer, liver diseases and autoimmune disorders (Veiga *et al.* 2020).

6.2.8 Other techniques worth mentioning

There are other ways of producing LNP than the methods described above. The methods are not as frequently used and are therefore not included in the report. However, the advantages and disadvantages of alternative methods e.g. Solvent Diffusion, Solvent Injection, Coacervation and Phase Inversion Temperature (PIT) will be listed in Appendix B.

6.2.9 Is one method more suitable than the other?

All the methods described in this section have their advantages and disadvantages, see Table 4. No method is suitable to produce all types of LNP since the desired properties and composition varies in different applications of the vesicles. The selection of production method influences the characteristics of the formed LNP. The loading efficiency of the drug to be encapsulated is also affected by the choice of production method.

The largest limitations of many of the described methods are low throughput and scalability issues. Due to the ability to scale-up and commercially available instruments, HPH and Microemulsion are the most common methods and also the most suitable for large-scale production of LNP. Microfluidic devices have yet to be tested for scale-up but have great potential in efficient and small particle formation.

Despite the environmental issues of the methods that include the use of organic solvent, they do have their advantages, the largest one being monodisperse particle formation. Methods like Supercritical Fluid Extraction evade the issues regarding polydispersity which leads to a significantly higher control of release kinetics and more efficient drug entrapment. If it's important that the dosing of the entrapped drug is precisely correct, monodispersity is essential and must be taken into consideration when choosing the appropriate method of synthesis.

Table 4. Overview of the advantages and disadvantages of different production methods of lipid nanoparticles (LNP), including High-Pressure Homogenization (HPH), Ultrasonication, Microemulsion, Solvent Evaporation, Supercritical Fluid Extraction and Microfluidic devices.

Production method	Mechanism	Advantage	Disadvantage	Reference
High-Pressure Homogenization	Strong turbulent eddies cause mechanical shear.	• Effective dispersing	• Energy intensive process • Polydispersity	(Ganesan & Narayanasamy 2017)
Hot High-Pressure Homogenization	Pressure drop through the instrument cause intense cavitations.	• Commercially available • High content of lipid • Reproducibility • Scalable • Well-established method	• Distribution of drugs into the aqueous phase • High energy input • Hydrophilic drugs distribute in the aqueous phase • Modifications of lipids and super-cooled melts • Reduction of efficiency due to heat	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015)
Cold High-Pressure Homogenization	Pressure drop through the instrument cause intense cavitations without high temperatures.	• No crystalline modifications due to increased temperatures • No drug degradation due to increased temperatures • Rapid cooling increases drug-loading suitable for hydrophilic drugs • Scalable • Thermo-sensitive drugs can be used	• Drug dischargement during storage • High energy inputs • Large particles	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015)
Ultrasonication	Cavitation causes formation and fricative collapse of globules.	• Low shear stress • Small particle sizes	• Contamination due to metal shading • High energy input • Relatively low drug entrapment efficiency • Scalability has not been tested	(Ganesan & Narayanasamy 2017)
Microemulsion	Emulsification and subsequent cooling forms particles.	• Avoids particle aggregation • Low energy input • No need for sophisticated equipment • Spherical particles • Theoretical stability	• High sensitivity to change • Labor intensive • Large volume of water creates strong dilution of LNP • Low LNP concentration • Polydisersity	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015, Saghazadeh *et al.* 2018)

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Production method	Mechanism	Advantage	Disadvantage	Reference
Solvent Evaporation	Solvent evaporation in an anti-solvent forms LNP.	• Easy to scale-up • Low energy input • No increased temperatures • Small particle sizes • System formed have low viscosity • Suitable for thermolabile drugs	• Decreased solubility of lipids in organic solvents • Emulsion instability • Low dispersion degree • Risk of particle aggregation due to slow evaporation • The organic solvent may be present in the final stage • Toxicological issues	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015)
Supercritical Fluid	Formation of LNP by supercritical fluid extraction of organic solvent from emulsions.	• Easy removal of impurities • Low temperature and pressure • Monodisperse particles • Particles as dry powder • Solvent removal is rapid and efficient • Stable LNP dispersions • Suitable for thermolabile drugs	• Expensive • Use of organic solvents	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015)
Microfluidic devices	Shear stress induced by laminar flow leads to particle formation.	• Can fabricate multicompartmental droplets • Enable double emulsion droplets • Reproducible and uniform LNP	• Can require subsequent removal of residual organic solvent • Limitation of geometry • Low throughput • Particles may be larger than those from emulsification	(Saghazadeh *et al.* 2018)

7 Purification of exosomes

The focus on this section will be on the exosomes only. Due to exosomes being distributed through complex body fluids, specific requirements are essential for the methods to be used in clinical applications. Vader *et al.* (2016) explains that the high yields are not the only property that is required, the applications must also be standardized and scalable, as well as taking capacity, reproducibility and purity into account. For instance, Ultracentrifugation is considered as the “golden standard” for exosome purification and Li *et al.* (2017) explains that this statement is based on the technique´s high processing capacity and low contamination risks with separation reagents. Today, there is no one-size-fits-all purification technique that can handle a variety of sample sources. On the other hand, due to progress in science and technology in the past years, many different techniques for purification have been developed. Each of the techniques exploit a specific trait, such as size, density and surface proteins. 20-X2 will therefore present five of the main exosome purification techniques in the following sections, but also a set of compared properties such as purity, capacity, ease of use, recovery, resolution, scalability, automatization, time commitment and special equipment, see Table 5. By analyzing the procedures and point out their advantages and disadvantages, 20-X2 will provide a wide view of the current

purification techniques, namely; Ultracentrifugation, Ultrafiltration, Size-Exclusion Chromatography, Immunoaffinity Capture and Microfluidic techniques.

Table 5. Exosome purification techniques.

Purification technique	Theoretical background	Advantage	Disadvantage	Reference
Ultracentrifugation	Size and density decide how fast it takes for the particles to settle out.	• Large sample capacity • Large yields	• High forces may ruin exosomes • Expensive equipment • Low portability • Low purity • Not suited for small samples • Time-consuming	(Gurunathan *et al.* 2019, Li *et al.* 2017, Yang *et al.* 2020, Witwer *et al.* 2013)
Ultrafiltration	Membranes separate particles based on their sizes.	• Cheap equipment • Easy to use • Fast procedure • Moderate portability	• Clogging in the membranes • Moderate purity	(Watson *et al.* 2018, Lobb *et al.* 2015, Li *et al.* 2017)
Size-Exclusion Chromatography	A porous stationary phase and the particle sizes affect which particles that will be eluted first.	• Fast procedure • High purity • High reproducibility • Native state is maintained • Small and large sample capacities • Uses all type of biological samples	• Moderate equipment cost • May need supplementary methods	(Batrakova & Kim 2015, Monguió-Tortajada *et al.* 2019, Nordin *et al.* 2015, Watson *et al.* 2018)
Immunoaffinity Capture	Exosomes are captured by utilizing the specific interactions that occur between immobilized antibodies and exosome tags (antigen).	• Easy to use • High purity • No chemical contamination	• Exosome tags must be added • Expensive reagent cost • Low sample capacity • Low yields	(Yang *et al.* 2020, Coumans *et al.* 2017, Monguió-Tortajada *et al.* 2019)
Microfluidic technologies	Isolation on microscale that can be based on properties such as size and immunoaffinity.	• Cost-effective • Easily automated • Fast procedure • High portability • High purity • Highly efficient	• Lack of standardization • Low sample capacity	(Liu *et al.* 2019, Woo *et al.* 2017, Batrakova & Kim 2015)

7.1 Ultracentrifugation - still considered as the “golden standard”?

To this day, ultracentrifugation-based purification are the most frequently used type of techniques. With their capacities to generate centrifugal forces up to 1 000 000 g, the techniques can separate particles based on density and size (Li *et al.* 2017). But why did this type of exosome purification get the label “golden standard” of all the different techniques? 20-X2 will discuss features and applications of the most regularly used version in this purification class below.

7.1.1 Procedure

Differential Ultracentrifugation is the most used exosome isolation technique according to Yang *et al.* (2020). The purification can be described as a cycle of multiple centrifugation steps where the centrifugal force increases for every cycle. Depending on the heterogeneity of the biological sample, a cleansing step may have to be done in order to remove large particles, such as cells. During such circumstances, low-speed centrifugation (> 400 g) is necessary. During forces around 2000 g, the pellets will mainly consists of cell debris. Meanwhile apoptotic bodies and protein aggregates settles out around 10 000-20 000 g and exosomes between 100 000-150 000 g. A schematic illustration of the workflow is presented in Figure 23. In addition, Witwer *et al.* (2013) emphasize that the particles in the biological sample are separated due to their physical properties, such as density, size and shape. As described above, bigger and denser particles settles out faster than smaller ones.

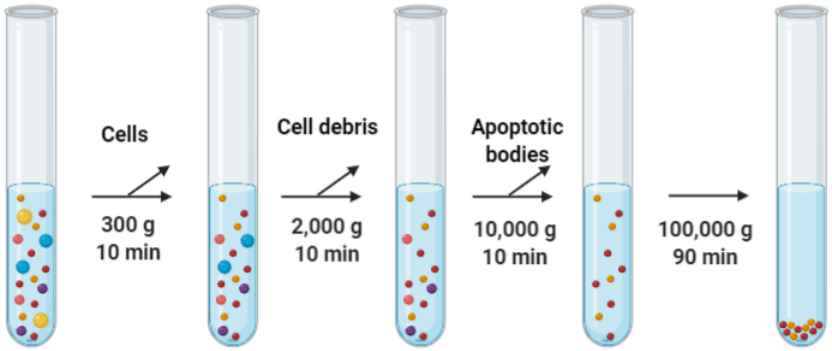


Figure 23. Schematic illustration of exosome purification with Differential Ultracentrifugation. The technique removes particles along a cycle consisting of multiple centrifugation steps. Depending on how high the centrifugal force is, different kind of particles are settled out from the sample. The pellets will mainly consists of cells (yellow) at 300 g, cell debris (blue) at 2000 g and apoptotic bodies (pink and purple) at 10 000 g. After the removal of the supernatant in the final step at 100 000 g, pellets containing both exosomes (red) and contaminant proteins (orange) can be collected.

7.1.2 Advantages

For the past three decades, Differential Ultracentrifugation has been used frequently around the globe to purify exosomes from various biological samples due to its compatibility of handling sample volumes between mL-L and lack of complex sample pretreatments (Yang *et al.* 2020). Witwer *et al.* (2013) brings up that the methods are suitable for pelleting extravesicular protein complexes and other contaminants.

7.1.3 Disadvantages

Although, Differential Ultracentrifugation has been around for a while, complications can occur, especially with high heterogeneity samples. The particles in a biological sample can reach a threshold and be precipitated during certain centrifugal forces. As a result, the exosome samples may contain contaminants which can complicate downstream applications. When it comes to recovery of exosomes, Lobb *et al.* (2015) reports that it has varied quite much between studies, namely; 2 % to 80 %. In addition, the technique has an ability to concentrate a

sample up to ∼ 8-fold (Coumans *et al.* 2017).when it comes to purification of exosome from clinical samples, Witwer *et al.* (2013) mentions several disadvantages. For instance, it is a time-consuming process that includes multiple repeatable centrifugation steps and Lobb *et al.* (2015) specifies that such a process takes around 3-9 hours. Furthermore, the repeatable steps conclude that the technique is not autonomous and the reappearing of centrifugal forces may damage the exosomes (Webber & Clayton 2013, Gurunathan *et al.* 2019).

Differential Ultracentrifugation can already handle sample volumes on a liter-scale and scaling up the capacity even more would be difficult. Mainly because the apparatus already are big and bulky but also because they are expensive, the personal need previous training due to operator sensitivity and the centrifugation time would become even longer. Nevertheless, even if it was manageable to increase the capacity, it would not solve the problems of handling contaminants such as protein and lipoprotein. This could easily ruin the samples (Yu *et al.* 2018, Monguió-Tortajada *et al.* 2019). Today, there is no easy way of handling contaminants that are of similar size as the exosomes. However, Coumans *et al.* (2017) and Gurunathan *et al.* (2019) suggest similar solutions for this problem and that is to use other purification methods such as Ultrafiltration and Size-Exclusion Chromatography. Gurunathan *et al.* (2019) continues pointing out that all above-mentioned disadvantages can create big complication when working with small clinical samples.

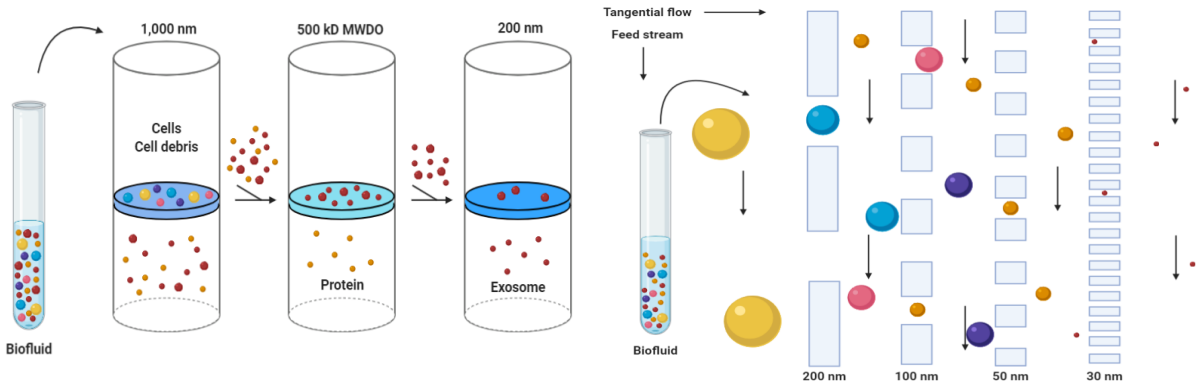
7.2 Ultrafiltration - the upcoming breakthrough in large scale purification?

This group of size-based purification techniques help people everyday without us knowing it. The technique helps us by cleansing our drinking water and blood through dialysis (Jönsson & Trägårdh 1990). This indicates that Ultrafiltration, as it is called, already exists as large scale in some areas. The purification procedure is as following; high forces such as high pressure push the samples through semipermeable membranes or molecular weight cut-offs (MWCO) (Gurunathan *et al.* 2019). However, the question is, can this be applied on purification of exosomes on a larger scale?

7.2.1 Advantages

Ultrafiltration-based exosome purification is much faster and easier to handle compared to ultracentrifugation-based methods primarily because the techniques do not require special apparatus. A normal procedure according to Lobb *et al.* (2015) and Watson *et al.* (2018), takes between 20 minutes to 1 hour when handling sample volumes between µL-L, which is much faster than 3-9 hours that was required for Differential Ultracentrifugation. Ultrafiltration-based techniques have ability to concentrate exosomes up to 240-fold and recovery up to 80 % (Lobb *et al.* 2015). However, Li *et al.* (2017) emphasize that the force that is created by the pressure may damage the particles (e.g. exosomes) that could potentially affect the downstream analysis. Li *et al.* (2017) also highlights that the interfering between particles are smaller in ultrafiltration-based methods compared to ultracentrifugation-based. Additionally, it has been proven that a better yield of recovery can be achieved because the particles do not squeeze or tackle each other as much.

According to Yu *et al.* (2018), ultrafiltration-based purification is an ideal substitute to ultracentrifugation-based. Especially for the easy adjustable membranes, that allows separation of specific subsets of exosomes with defined sizes (Heinemann & Vykoukal 2017). Moreover, multiple Ultrafiltration devices have been developed to provide a greater field of more manageable candidates that can give faster preparation with better yields (Yang *et al.* 2020). Two Ultrafiltration procedures are illustrated in Figure 24, namely; Sequential Ultrafiltration and Tangential Flow Filtration.



(a) Sequential Ultrafiltration. *(b) Tangential Flow Filtration.*

Figure 24. Schematic illustration of two ultrafiltration-based techniques for exosome purification. (24a) Sequential Ultrafiltration. When the biological fluid cross the 1000 nm membrane, larger particles such as cells (yellow), cell debris (blue) and apoptotic bodies (pink and purple) are removed. The remaining particles in the sample passes through a 500 kD MWCO, which only let free proteins (orange) through. The particles that were captured in that membrane will now pass a 200 nm membrane, exosomes smaller than 200 nm (red) cross the membrane and are collected. (24b) Tangential Flow Filtration. Parallel to the membranes (gray) flows a feed stream and some of this stream will pass through these membranes due to a high pressure. The membranes have different filter sizes and particles will pass through the membrane with the feed stream if their sizes allow them to. Particles larger than 200 nm, such as cells (yellow), cannot cross the first membrane meanwhile exosomes (red) can pass through all the membranes and be collected at the end.

7.2.2 Sequential Ultrafiltration

The first method that was mentioned in Figure 24 was Sequential Ultrafiltration. The exosomes are purified by letting the sample cross several membranes that are connected in a series. Every membrane have a unique MWCO that can separate different cells from each other. Cells and cell debris were the first type particles to be excluded at 1000 nm membrane in the above-mentioned example meanwhile proteins where the next type to be removed at 500 kD. The exosomes can be concentrated in the last step by using a 200 nm membrane (Popović & de Marco 2018). As a result, the exosomes are isolated with a high purity. Despite having higher purity than ultracentrifugation-based methods, this method also has its limitations. One of the biggest problem is that the particles can clog the membranes, which may lead to a lower separation yield and a shorter lifespan of the membranes (Gurunathan *et al.* 2019). Just like the ultracentrifugation-based techniques, the biggest contaminant are similar-sized particles, but the technique also have problems with non-exosomal proteins. There have been reports that claims; combinations of different MWCO along with Size-Exclusion Chromatography can remove these non-exosomal proteins (Shu *et al.* 2020).

7.2.3 Tangential Flow Filtration

However, the technology expand and researchers finds new ways to go around the clogging problem. For instance, Tangential Flow Filtration reduces potential clogging by manipulating a permanent flow force that migrate parallel to a module of hollow fiber membranes (Monguió-Tortajada *et al.* 2019). The pressure causes some of the flow stream to move across the membranes. In addition, the membranes have different filter sizes and particles will pass through if they have an appropriate size. Yang *et al.* (2020) points out that the remaining particles in the module can be recirculated back to the beginning of the process. This ensure a thorough filtration as well as an automated procedure with higher yield. However, like every other size-based purification method Tangential Flow Filtration can not guarantee a completely contamination-free sample. Nevertheless, the technique still has a significant ability of removing protein and other particles (Watson *et al.* 2018). Due to this trait, Tangential Flow Filtration has a high potential of separating exosomes from many different clinical samples.

7.3 Size-Exclusion Chromatography - preserver of the biological activities

Another popular size-based purification technique that have been around for decades is Size-Exclusion Chromatography (SEC). It is a relatively simple and well-developed chromatographic method that isolate particles in fractions. Today, the technique is widely applied when it comes to high-resolution isolation of proteins and liposome particles (Yang *et al.* 2020).

7.3.1 Procedure

SEC consists of a column containing a firm packed stationary phase made of polymer beads that contains pores in different sizes. These pores can be on the surface or go through the bead, which makes it possible to sort particles based on their size. The separation is possible by passive gravity flow and when a biological fluid passes through the stationary phase particles with small radii are able to enter the pores. This results in longer traveling distance and a late elution. On the other hand, particles with large radii cannot enter as many pores like the smaller particles, therefore, are they eluted much earlier (Li *et al.* 2017), see Figure 25.

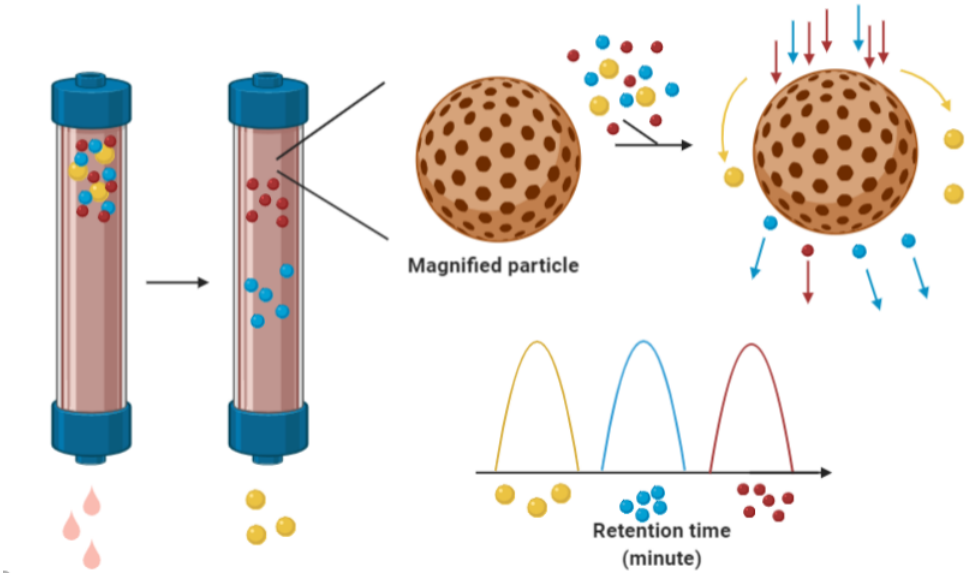


Figure 25. Schematic illustration of exosome purification with Size-Exclusion Chromatography. When a biological fluid passes through a porous stationary phase, particles are separated based on their sizes. Particles that have smaller radii than the pores of the stationary phase can enter these pores and get slowed down in the narrow paths. Meanwhile larger particles have to move around the resin, causes them to move faster out of the column and have the fastest retention time.

7.3.2 Advantages

Today, many different stationary phases exists, all have a specific range of separation. However, columns packed with Sepharose CL-2B is, according to Monguió-Tortajada *et al.* (2019) the most used matrix with successful exosome purification. The authors continue to highlight that the method is the most promising one when it comes to purifying exosomes from all type of biological fluids and that the technique can remove overabundant soluble plasma proteins. Because the technique avoids unnecessary co-isolations of contaminants, generally not need preparations and the collection of exosome is quite simple (Yang *et al.* 2020). Moreover, the matrix provide a higher recovery and purity compared with Differential Ultracentrifugation. This manageable technique can be used in "fingertip" analysis and it is easily adaptable to most laboratories. This results often have a high-resolution (Stranska *et al.* 2018). Additionally, the whole process can be done in a reasonable time (e.g. 20 minutes) (Böing *et al.* 2014). Lastly, SEC achieve a higher yield compared to other size-based purification method such as Ultrafiltration, mainly because the solutes and the stationary phases do not interfere with each other (Gámez-Valero *et al.* 2016).

7.3.3 Disadvantages

Even though this technique is easily scalable and automated it still has it limits. For instance, Coumans *et al.* (2017) mentioned that the column height and the pore size affect the techniques performance. Due to SEC uses gravity flow, a longer run time is necessary (Batrakova & Kim 2015). This limits the technique scalability for high-throughput applications. The technique also have the same problem with similar-sized contaminant as ultracentrifugation-based and ultrafiltration-based, especially larger protein aggregates and lipoproteins. This

could potentially affect the final exosome sample. However, SEC can remove the majority of these contaminants and preserves the exosomes native state due to passive gravity flow and that SEC has excellent reproducibility (Diaz *et al.* 2018). Anyhow, to eliminate the contaminants that were mention above several researchers have combined SEC with ultrafiltration-based techniques (Shu *et al.* 2020). This kind of combination have shown to have an increased ability to collect more exosomes with an improved purity as well as preserve the exosomes biological functions.

7.3.4 Advantages with Size-Exclusion Chromatography and Ultrafiltration in combination

In a Swedish study that was performed by Nordin *et al.* (2015) tested the above-mentioned combination of SEC and Ultrafiltration. It resulted in a significantly higher yield and the composition of the vesicle proteins were not affected. For instance, a combination of Ultrafiltration and SEC recovered 25 % more of the total amount of CD63-eGFP molecules compared to Ultracentrifugation. In addition, the combination also had high recovery rates (70 % ± 19 %).

SEC alone also has difficulties with concentrating samples. Monguió-Tortajada *et al.* (2019) suggest that the sample should be concentrated before the loading. One example is to use Tangential Flow Filtration because the technique can handle large volumes and avoid clogged membrane. This was also brought up by Watson *et al.* (2018). The study says that Tangential Flow Filtration and SEC can process large starting volumes without losing yield. Watson *et al.* (2018) compared how much purer a sample got on a larger scale when Tangential Flow Filtration was combined with SEC in contrast to SEC. The column had the dimensions; 60 cm length, 1,6 cm diameter and 120 mL bed volume. As result, the amount of contamination decreased by a 10-fold when Tangential Flow Filtration was used.

7.4 Immunoaffinity Capture - the finder of all subgroups?

A property that all cells have in common is that their membranes contain plenty of receptors and other proteins. This also applies to exosomes resulting in great opportunities regarding the development of purification techniques with high specificity. Immunoaffinity Capture methods exploit interactions that arise between the membrane-bound proteins (antigens) and the corresponding antibodies (Li *et al.* 2017).

7.4.1 Procedure

During the past decades, several exosome tags have been found and they are being used as antigens. The chosen antigens are common and specific just for exosomes. For instance, antibodies are mainly used against Ras-related proteins such as RAB5 and tetraspanins such as CD9, CD63 and CD81. Exosome purification can be done by exploiting one tag or a combination of many. This results in a total exosome isolation from a biological sample (Monguió-Tortajada *et al.* 2019, Yang *et al.* 2020). Furthermore, the antibodies are often covalent linked to beads or other matrices, including chromatography, plates and in several different microfluidic techniques (Witwer

et al. 2013). The most frequently used immunoaffinity strategy according to Yang *et al.* (2020) is magnetic particles in immuno-precipitation, see Figure 26.

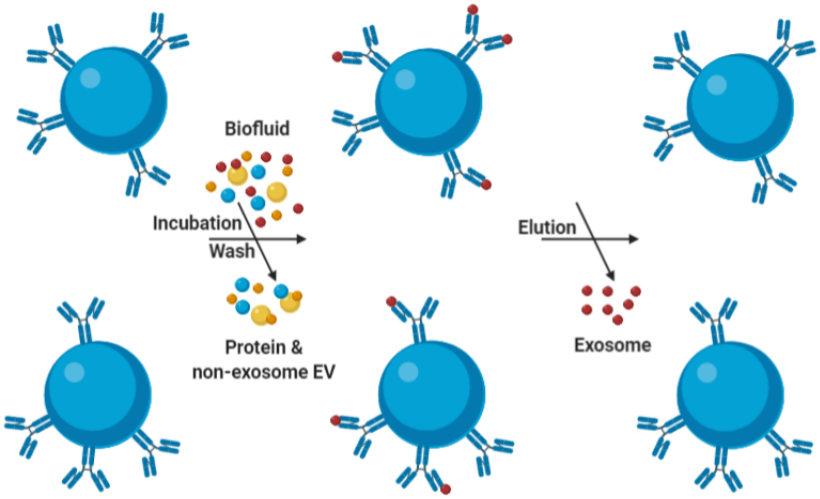


Figure 26. Schematic illustration of immunoaffinity-based exosome purification. After incubation of a biological fluid, antibodies (Y-shape) on the solid phase (blue) binds with the specific tags that are located on the exosomes´ (red) surface and immobilizes them. Meanwhile protein (orange) and other particles (yellow and blue) are washed away. Exosomes are then collected after an elution step.

7.4.2 Advantages

Gurunathan *et al.* (2019) emphasize that this type of technique is especially useful when the membrane-bound proteins miss a soluble counterpart. Additionally, the technique can also purify and enrich exosomes from various biological samples such as biological fluids, cell culture and tissues. In comparison with Differential Ultracentrifugation, Immunoaffinity Capture have a greater ability to concentrate exosomes, namely up to 10-15 times higher (Gurunathan *et al.* 2019). Immunoaffinity Capture still has the upper hand when it comes to ease of use and compatibility with routine equipment. The above-mentioned technique has the ability to achieve a high sensitivity as well as high efficiency. Further, the method can handle sample volumes between µL-mL (Li *et al.* 2017).

7.4.3 Disadvantages

Despite several promising advantages, this technique also has its disadvantages. The biggest limitation is to find antibodies with high specificity for an antigen of interest. For instance, there are still no fully deterministic exosome tags. This can create “noise” from other particles and proteins, especially from soluble proteins because they belong to a major contamination group (Monguió-Tortajada *et al.* 2019). The elution buffers that are used to separate exosomes from the antibodies can damage the biological function of the exosomes so bad that the effect is irreversible. This could potentially affect exosome-based studies as well as therapeutic applications (Yang *et al.* 2020). Batrakova & Kim (2015) and Coumans *et al.* (2017) reports that this approach is time-consuming and that it results in much lower yields because the specificity of the antibodies only recognize subsets of all the

vesicles in the samples. However, Immunoaffinity Capture is thereof one of the method with the highest resolution. Furthermore, Immunoaffinity Capture is one of the most expensive methods of all the presented techniques. Primarily of the highly specific antibodies and these aspects can be a problem in clinical applications (Li *et al.* 2017).

7.5 Microfluidic technologies - the next generation purification strategy

New techniques are essential to cope with the challenges of clinical applications. Methods such as Ultracentrifugation faces several challenges such as high cost, low yield and purity. Luckily, the area of microfabrication technology has exploded in this decade and offers today a variety of exosome purification devices at microscale with combined detection and analysis apparatus. These lab-on-a-chip microfluidic systems have the ability of exploiting both physical and biochemical features of exosomes such as size and immunoaffinity. However, new separation mechanisms have been developed and they are based on acoustic, electrophoretic and electromagnetic manipulations (Liu *et al.* 2019).

7.5.1 Procedure

The lab-on-a-chip format simplify the purification by combining several different processing instruments. This results in a reduced risk of cross-contamination (Contreras-Naranjo *et al.* 2017). A microfluidic chip often consists of an inlet, a separation region and an outlet. The biological fluid is added into the inlet, thereafter, the fluid is transported to the separation region. This part of the device is made of microchannels or microstructures. Particles at this location are expose for fast transportation as well as precise manipulations. After the separation, the exosomes continue to a combined detection and analysis apparatus at the outlet that can be used for real-time classification for *in situ* diagnosis, see Figure 27.

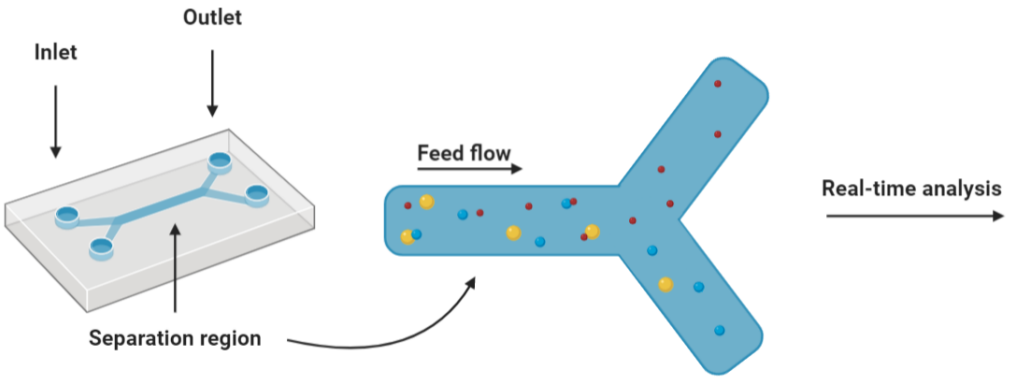


Figure 27. Schematic illustration of a microfluidic exosome isolation chip with combined analysis. When a biological fluid is added onto the inlet, exosomes (red) and other particles (blue and yellow) can be separated by their physical and biochemical properties in the separation regions. After the separation can real-time exosome analysis for in situ diagnosis be executed.

7.5.2 Advantages

Although, this kind of technique is at an early-stage of development, it still holds great potential in rapid-screening tools in clinical applications. Especially for the high purity, fast processing as well as the use of small sample volumes. But also for the low cost, a single chip costs in total between $2–$5 (Batrakova & Kim 2015, Dorayappan *et al.* 2019), which is cheap compared to techniques such as ultracentrifugation-based (Gurunathan *et al.* 2019). However, this merge of steps make microfluidic-techniques cost-effective. The processes take a maximum of 2 hours (Chen *et al.* 2019), in some cases the procedure only takes 15 minutes (Liu *et al.* 2019).

Microfluidic system with integrated immunoaffinity is a frequently used strategy. Antibodies on the surface of the devices´ separation region can capture, for example, the tetraspanin CD9, CD63 and CD81 that are located on the surface of the exosomes (Yang *et al.* 2020). Micropillar arrays functionalized with multiwall carbon nanotubes (MWCNTs) is a rapid exosome purification technique that has an exosome recovery above 80 % when the flow rate was set to 1 mL h^{-1} (Wang *et al.* 2017). However, the separation can only be executed when the exosomes have tags added to them. Another purification strategy is by using membrane and one example of that is an Exodisc (Woo *et al.* 2017). The separation region consists of double filtration systems. The device achieved a high recovery yield of exosomes and removed more than 95 % of all contaminants. Furthermore, exosomes can be separated by size by applying external physical fields. For instance, a device that separated exosomes and other particles with acoustic fields had a recovery and purity of 82,4 % respectively 98,4 % (Wu *et al.* 2017).

7.5.3 Disadvantages

Even though Liu *et al.* (2019) say that this kind of technique will be “the new era of cancer diagnostics and therapy” and that a massive progress have been made. The first generation of microfluidic devices still has its flaws which stop them from being used frequently in clinical trials. They have issues with scaling, standardization as well as time-consuming sample preparations. Even though the examples above show high results in both purity and recovery, the devices could have had higher yield and specificity as well if they did not use just one sorting mechanism. To overcome this disadvantage, a potential strategy involves combining several different sorting mechanism into one integrated device. In addition, the low processing capacity can disrupt downstream analysis due to poor amounts of nucleic acids and proteins (Li *et al.* 2017).

7.6 Which purification method is most optimal

The development of exosome purification techniques have made a tremendous progress during the past decade. There are still fundamental questions that have not been answered yet. Which results in some challenges despite the progress, especially when exosomes are purified quickly from a complex biological sample, the heterogeneity of exosome as well as overlapping of different vesicles biochemical and physical properties (Li *et al.* 2017).

Even though Differential Ultracentrifugation is currently considered, as the “golden standard” of the exosome purification techniques and that the apparatus has a handle large sample capacity. The isolated exosomes are often purified along a large amount of protein and lipoprotein contaminants, which could easily ruin the sample. In addition, purification procedure is time-consuming and requires trained staff (Lobb *et al.* 2015, Webber & Clayton 2013).

Despite ultrafiltration-based techniques being easier to handle and can isolate exosomes with a higher purity than the “golden standard” the techniques have limitations as well. One of the biggest is that the membrane clogs which ultimately reduces the lifetime of the membranes and the isolation efficiency. This could potentially create errors in test results (Gurunathan *et al.* 2019). Luckily, this problem can be handled by Tangential Flow Filtration. However, the techniques has still its limit similar-sized contaminants and moderated processing volume (Monguió-Tortajada *et al.* 2019).

SEC however, has shown some unique advantages such as removing overabundant soluble plasma proteins which Differential Ultracentrifugation cannot do (Nordin *et al.* 2015, Watson *et al.* 2018). This results in a purer recovery of exosomes. In addition, the procedure is short and it does not requires special training to handle to equipment. Due to good reproducibility and high quality exosome preparation, Yang *et al.* (2020) believe that the technique may have potential in high throughput industrial applications. Especially for the use of gravity flow that reduces the damage on the exosome. However, the gravity flow is a roadblock when it comes to the scaling ability. The higher the column is the longer run time is necessary. This can turn into a time-consuming process (Batrakova & Kim 2015).

Another technique that can be quite time-consuming is Immunoaffinity Capture and despite having one of the highest specificity of all technique, there are still no fully deterministic exosome tags to capture these antigens with high specificity. As a result, “noise” is created from other particles and proteins (Monguió-Tortajada *et al.* 2019). This could potentially affect the result in clinical testing. In addition, the high specificity leads to lower yields because the antibodies only recognize subsets of all the vesicles in the samples (Batrakova & Kim 2015, Coumans *et al.* 2017).

Although, microfluidic devices have made a great progress the last years, this type of technique has still not been applied in clinical applications. The reason why it has not come longer according to Liu *et al.* (2019), is the lack of standardization and scalability. Yang *et al.* (2020) believe that microfluidic techniques have a chance of being applied in clinical application in the future if improvements are made in their capacities. Especially through multiple exosome sorting mechanisms and large setups of parallel microfluidic devices.

Table 6 mentions nine properties that are compared with the five purification techniques. *Purity* indicates how good the method can separate the exosomes from contaminants. *Scalability* shows how easy it is to scale-up a process without increasing the costs or time. *Automatization* rates how easy it is to automate a process. *Recovery* refers to how big the exosome yields can be. *Ease of use* indicate if specific training is needed and how difficult it is to operate the techniques. *Time commitment* shows long time it takes to process a sample. *Capacity* presents how large the sample volume can be. *Resolution* rates how well the technique can separate different exosome

populations. *Special equipment* shows if specialized non-consumable equipment is needed.

Table 6. Property comparison of the exosome purification techniques.

	Ultracentrifugation	Ultrafiltration	Size-Exclusion Chromatography	Immunoaffinity Capture	Microfluidic technologies
Purity[1]	1	2	2	3	3
Scalability[2]	1	2	3	2	1
Automatization	No	Yes	Yes	Yes	Yes
Recovery[1]	2	2	3	1	2
Ease of use[2]	2	3	3	2	2
Time commitment	3h - 9h	$\sim$ 0.3h	$\sim$ 0.5h	4h - 20h	$<$ 2h
Capacity	mL-L	µL-L	µL-mL	µL-mL	µL
Resolution[1]	1	1	2	3	3
Special equipment	Yes	No	No	No	Yes

In conclusion, there is no technique today that is “perfect” to purify exosomes. All techniques have different disadvantages depending on what property they exploits of the vesicles. The one-size-fits-all model may occur in future purification devices. However, some important features that must be taking care of if they are going to be used in clinical application is low contamination and isolation of specific subpopulations (Shu *et al.* 2020). Even though both Immunoaffinity Capture and Microfluidic technologies generates the highest purity of all the mentioned purification techniques. These two have the same problems when it comes to scalability, which means that it will take a longer time to get a certain amount of exosomes. Especially for Immunoaffinity Capture because it has a low recovery rate and the process take multiple hours. Ultrafiltration-based techniques and SEC have similar results in Table 6. The reason why Ultrafiltration has got a lower score was due to the problem with clogging. This means, according to 20-X2, that SEC alone is the most suitable technique today due to its scaling possibilities, easily adaptable equipment and low breakage of exosomes. However, several studies point out that combinations of SEC and ultrafiltration-based methods can achieve a more purified sample of exosomes as well as the biological functions of the exosomes being preserved (Nordin *et al.* 2015, Watson *et al.* 2018).

[1] 1 ($<$ 30 %) to 3 ($\sim$ 90 %)
[2] 1 (low) to 3 (high)

8 Detection and analysis of exosomes and lipid nanoparticles

When producing exosomes and lipid nanoparticles (LNP) for drug delivery it is important to be able to analyze the results from production and purification steps. To date there are no standardized methods for detection of exosomes or LNP but there are a lot of methods developing and on the market with potential of both detection and characterization of the vesicles. In this section different techniques and problems of detection will be presented. A table comparing the different methods is also displayed in Section 8.3.

8.1 Identifying characteristics

There are different identifying characteristics to look for when trying to detect the different types of vesicles. In exosomes one of the most important characteristics to look for is surface components (Johnsen *et al.* 2014), for example proteins. Different proteins are enriched in exosomes (Conde-Vancells *et al.* 2008) making it possible to be more specific in detection and exclude other types of vesicles. Exosomes are also defined by size and lipid composition (Johnsen *et al.* 2014). Similar as for exosomes, size is an important feature for LNP (Shah *et al.* 2015). Another aspect that is important to examine for LNP described by Shah *et al.* (2015) is if the sample is monodisperse, meaning that the particles have the same size. It is important to have a monodisperse sample of LNP because it affects characteristics of melting, control of release kinetics and drug encapsulation efficiency (Rawat *et al.* 2008). An additional important property for both types of vesicles is concentration. Concentration of exosomes is correlated with when to harvest the cells in production (Patel *et al.* 2017).

8.1.1 Surface components and fluorescence are important for detection of exosomes

As described, one important feature when detecting exosomes is the surface components. Exosomes are heterogenic, and different vesicles can contain different compositions and amounts of surface components (Tian *et al.* 2018a). These differences are responsible for variation in functionality and thereby the interest in using the vesicle (Penders *et al.* 2018), which makes it important to be able to detect the differences. There are labeling techniques enabling detection that can be used. These techniques often use binding complexes where one example is IgG-fluorophore complex (Buzás *et al.* 2017) and another is DNA ligated with fluorescing molecules (Löf *et al.* 2016). There are difficulties in these processes, for example that the IgG-fluorophore complex is big in comparison with exosomes making binding between these components harder and limiting number of molecules that can bind (Buzás *et al.* 2017). This can lead to a signal that is too weak to detect (Rupert *et al.* 2014). Moreover, it is also important to clear out all un-bound labels which require further filtration steps according to Rupert *et al.* (2014). Even though there are difficulties it is a prospect of exosome and exosome subpopulation detection, and these techniques are used in many applications and methods.

8.2 Many different detection techniques used

There are a lot of different techniques used to detect and/or measure the surface components, size and concentration in different ways. Examples include measuring amount of lipids (Sawada *et al.* 2020), optical measurement (Vogel *et al.* 2011, Rupert *et al.* 2014, Ha *et al.* 2016, Buzás *et al.* 2017, Pick *et al.* 2018), electron microscopy measurements (Vogel *et al.* 2011, Marquele-Oliveira *et al.* 2016, Pick *et al.* 2018, Lv *et al.* 2018), electrical impedance measurement (Vogel *et al.* 2011, Rupert *et al.* 2014, Buzás *et al.* 2017, Pick *et al.* 2018), mass determination (Abramowicz *et al.* 2016, Ha *et al.* 2016) and immunostaining (Lv *et al.* 2018).

The different techniques for detection and analysis used on the market today uses two main ways to provide information about the sample, either bulk or single-vesicle analysis. Conventional techniques use bulk analysis, which provide information about for example average size (Penders *et al.* 2018). This can be useful, but the number of methods developed for single-vesicle analysis is increasing. One reason for this is that the heterogeneity in size and other features between different exosomes makes it problematic to use conventional bulk analysis techniques. The reason why these average measurement results obtained from bulk analysis is not favorable is that different features have an impact on the functionality and the interest in using the exosome (Penders *et al.* 2018).

8.3 Comparison between different methods

In this chapter five different analysis methods are presented. All of them have different limitations and advantages that can be good to take into consideration when choosing a method for detection. In Table 7, the detection methods are presented in gray and platforms, assays or specific techniques using this method are presented in white underneath. The *Separation method* column is describing what type of method is mainly used for separating and detecting exosomes or LNP. *Measurement* is describing what type of measurement that can be obtained from the method and *Target* is describing if there is a targeted molecule, and in that case which have been used. *Detection time*, *Working concentration* and *Detection speed* describes different features that can be good to take in to account when evaluating the detection method. The column *Minimal detectable size* contains information about the minimal detectable size of vesicle that can be obtained. This is important both to detect all exosomes and to get a correct concentration measurement. The different techniques are described in more detail in Sections 8.3.1-8.3.5.

Table 7. Description of important properties of detection methods including Nanoparticle Tracking Analysis (NTA), Flow Cytometry, Surface Plasmon Resonance (SPR), Resistive Pulse Sensing (RPS) and Raman Spectroscopy, shown in gray header. Different methods and platforms using the header technique are shown in white. A similar table can be found in the article by Wang *et al.* (2020).

Technology/ Method	Separation method	Measurement	Target	Detection time	Working concentration [particle/mL]	Detection speed	Minimum detectable size	Reference
Nanoparticle tracking analysis	Microfluidics	Size, concentration, refractive index	-	-	-	-	70-90 nm	(van der Pol *et al.* 2014)
-	Fluorescent tracking	Size, concentration	Exo surface proteins	$\sim$ 0.3h	10^6-10^9	20-60/view	$\sim$ 50 nm	(Carnell-Morris *et al.* 2017)
-	Fluorescent tracking	Size, concentration	miR-21	$\sim$ 2.5h	10^{11}	100/view	$\sim$ 100 nm	(Baldwin *et al.* 2017)
Flow Cytometry	Microfluidics	Size, concentration	-	-	-	-	-	-
Conventional	Microfluidics	Size, concentration	Usually no target	-	$\sim 10^5$	-	270-600 nm	(van der Pol *et al.* 2014)
HCR engineering EV	Microfluidics	Quantification	CD63, HER2	$\sim$ 8.6h	$\sim 10^9$	Thousands particles/s	500 nm, EVs enlarged to overcome this threshold	(Shen *et al.* 2018)
A multi-color *in situ* proximity ligation assay	Microfluidics	Concentration	CD26, CD10, CD13, CD114 and Cathep-sin B	$\sim$ 4.5h	200 pg/mL-200µg/mL	10^3-10^4 particles /min	500 nm, EVs enlarged to overcome this threshold	(Löf *et al.* 2016)
High sensitivity Flow Cytometry	Microfluidics	Size, concentration	CD9, CD63, CD81, CD13 CD147, CD10	$\sim$ 4.6h	$5*10^9$ particles/mL	10^4 particles /min	40 nm	(Zhu *et al.* 2014, Tian *et al.* 2018a)
Surface Plasmon Resonance	Light capture	Size, concentration	-	-	-	-	-	(Wang *et al.* 2020)
nPLEX	Light capture, protein assay	Concentration, protein profile	EpCAM, CD24, CD63	$\sim$ 1 h	10^{11}-10^{12} particles /mL	-	-	(Im *et al.* 2014)
LSPRi/ nanoplas-monic pillars	Light capture, micro-machined chip	Digital responses	CD63	2 h	$\sim 10^5$ particles/mL	-	-	(Raghu *et al.* 2018)
Resistive Pulse Sensing	Tuneable nanopore	Size, concentration, charge of individual EV	-	$\sim$ 0.25-0.5 min	$\sim 10^7$-10^8	6000-12000 particles/h	70-100 nm	(Vogel *et al.* 2011, Maas *et al.* 2014)
Raman Spectroscopy	Light capture	Size, concentration, chemical composition	-	-	-	-	50 nm	(Bendix & Oddershede 2011)

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Technology/ Method	Separation method	Measurement	Target	Detection time	Working concentration [particle/mL]	Detection speed	Minimum detectable size	Reference
LTRS	Light capture	Chemical composition and concentration	-	~ 75 min	10^9*10^{11}	12 particles/h	100 nm	(Smith *et al.* 2015)
SERS	Light capture, exosomes absorbed on surface	Vesicle identification, chemical profile	-	~ 15 min	≤ 5*10^{10} particles/mL	7200 particles/h	-	(Stremersch *et al.* 2016)
SPARTA	Light capture	Size, composition, inter and intra particle population	-	~ 60 min	10^{10}-10^{12} particles/mL	360-720 particles/h	-	(Penders *et al.* 2018)

8.3.1 Nanoparticle Tracking Analysis - one of the most developed methods

Nanoparticle Tracking Analysis (NTA) is described as one of the most developed methods for detection of exosomes (Wang *et al.* 2020). It can also be used for other types of nanoparticles and extracellular vehicles (EVs). This technique measure the light scattering of individual nanoparticles present in a solution (Rupert *et al.* 2014), see Figure 28. NTA measures in real-time and can use different labeling to detect subpopulation of exosomes. When detecting exosomes a laser beam is focused into the test-solution, illuminating the particles (Buzás *et al.* 2017). The particles in the solution move in Brownian motion (Saveyn *et al.* 2010), and a video of the particles moving is recorded. Analysis of the video can be made and thereby it is possible to calculate the radius of the particle. Furthermore, the refractive index can be determined for each particle by measuring the intensity of the scattered light. NTA is described by Buzás *et al.* (2017) to be a good method for fast detection and assessment of the size, refractive index and approximate concentration of exosomes.

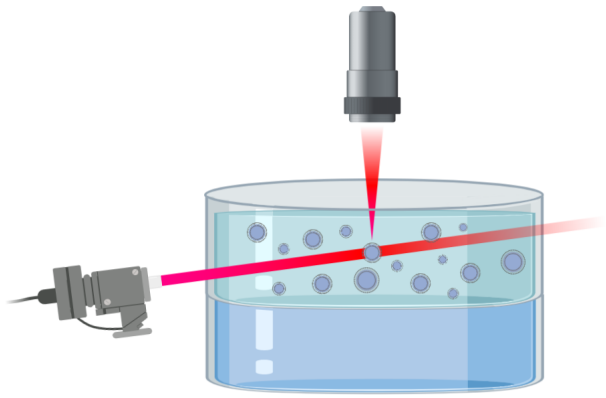

Figure 28. Schematic illustration of Nanoparticle Tracking Analysis, with a laser beam going through a sample with vesicles. The sample is placed on top of glass and a microscope is detecting the scattered light.

8.3.1.1 Difficulties in detecting exosomes

One difficulty with NTA is that due to the heterogeneity in size of exosomes, measurements have to be performed with different dilutions and detection settings to increase effectiveness (van der Pol *et al.* 2014). The insecurity of the diffusion coefficient, used in calculations, is one of the contributing factors for the uncertainty of this method (van der Pol *et al.* 2014). There is a high deviation in both size and concentration measurements compared to other methods (van der Pol *et al.* 2014). The minimum detectable size for this method is 70-90 nm (van der Pol *et al.* 2014) and the threshold for concentration measurements is 10^8-10^9 EV/mL per minute (Wang *et al.* 2020).

8.3.1.2 Possibilities and fluorescent use for exosomes

This technique have the possibility to detect fluorescent labels which enables it to phenotype exosomes and detect subpopulations (Buzás *et al.* 2017). The detection of phenotypes and subpopulation is more informative than only size and concentration measurements. According to Buzás *et al.* (2017) it is more difficult labeling with antibodies than detecting exosomes. Potential other labels that can be used are aptamers, affibody molecules or nanobodies.

When working with fluorescent labels, different targets can be used. Two examples are to use surface proteins (Carnell-Morris *et al.* 2017) or cargo miRNA (Baldwin *et al.* 2017). These different techniques have different advantages and disadvantages, for example that the method using surface proteins is much faster than the method using miRNA, or that the miRNA method needs less sample for detection, see Table 7.

8.3.1.3 Advantages regarding lipid nanoparticles

An advantage regarding LNP is that the time required for measurement is reduced if the sample is monodisperse (Saveyn *et al.* 2010). A monodisperse sample is desirable when producing LNP for drug delivery because of the impact it has on melting, control of release kinetics and drug encapsulation efficiency (Rawat *et al.* 2008).

8.3.1.4 Commercialized instrument and developed method

The instruments needed to perform NTA are commercialized and the software is developing more and more. Even though NTA is one of the most developed methods for detection of EVs it is limited to be used to characterization of particle size and concentration measurement according to Wang *et al.* (2020).

8.3.2 The role of Flow Cytometry is stated to increase

Flow Cytometry is largely used for detection of exosomes and LNP. The method is based on light scattering (Picot *et al.* 2012) and enables measurements of single particles. The sample is focused through a small pipe to allow for the vesicles to pass through a laser beam individually, see Figure 29. The scattered light can then be detected and analyzed (Picot *et al.* 2012). It is also possible to detect fluorescing molecules, which enables for different labeling to be used (Pick *et al.* 2018). The calculations from light scattering give insight to size and concentration of vesicles in the solution (Wang *et al.* 2020). There are also possibilities to detect different

subpopulations of vesicles through cell sorting based on biochemical or biophysical properties (Picot *et al.* 2012).

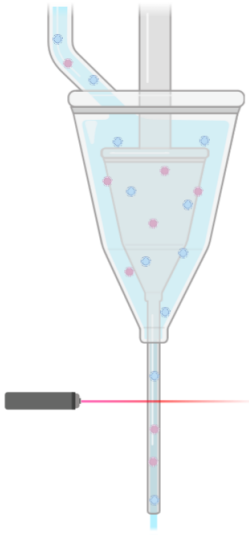

Figure 29. Schematic illustration of Flow Cytometry. A laser beam going through the pipe containing the sample with vesicles. The vesicles pass the light one by one. The scattered light can be detected and analyzed.

8.3.2.1 Different kinds of Flow Cytometry have differernt advantages and disadvantages

There are different types of Flow Cytometry with different limits for detection of vesicles. A Conventional Flow Cytometry has the minimal detectable vesicle of 270-600 nm while a Flow Cytometry Dedicated for Detection of Submicrometer Particles have a detection limit of 150-190 nm (van der Pol *et al.* 2014). Since most exosomes are in the range of 30-200 nm (van der Pol *et al.* 2014, Vlassov *et al.* 2012), the Conventional Flow Cytometer would not detect most of the vesicles which can make the method unsuitable for detection. Further, van der Pol *et al.* (2014) mention that the small number of antigens on the surface of exosomes in combination with big binding complexes, which makes it possible only for a few fluorescent labels to attach, can make it difficult to detect EVs using Flow Cytometry.

There are ways to overcome the difficulties with using Flow Cytometry. For Conventional Flow Cytometry, exosomes can be enriched with latex beads or aptamers (Wang *et al.* 2020). The aptamers can be amplified using Hybridization Chain Reaction (HCR). The use of HCR is described by both Löf *et al.* (2016) and Shen *et al.* (2018), where Löf *et al.* (2016) used an assay called ExoPLA. In these articles' antibodies conjugated with DNA oligonucleotides were described to bind to proteins on the surface of the exosomes and then the DNA was amplified. The exosomes could thereafter be detected by fluorescent labeled detection DNA oligonucleotides added to the complexes formed in the reaction. The advantage with this according to Shen *et al.* (2018) is that Conventional Flow Cytometers, that might already be present in many labs, can be used to detect exosomes even though the minimal detectable vesicle is about 500 nm. In the assay described by Löf *et al.* (2016) five different antibodies are used, which makes the detection have a high specificity towards exosomes.

8.3.2.2 Can High Sensitivity Flow Cytometry be the future?

The role of using High Sensitivity Flow Cytometry for detection of exosomes and other nanoparticles is stated to

increase (Wang *et al.* 2020). In this method exosomes down to 40 nm can be detected (Tian *et al.* 2018a). Fluorescent labels can also be used. These detections can be made within a few minutes (Wang *et al.* 2020), which is an advantage with this method compared with other methods even though the whole process is still time-consuming.

8.3.3 Surface Plasmon Resonance is a high-sensitivity detection tool

Surface Plasmon Resonance (SPR) can be used for accurate detection of exosomes (Raghu *et al.* 2018). The method is based on binding events between two molecules, one attached to a surface and the other flowing past the surface in solution (Rupert *et al.* 2014). A laser is focused on the other side of the surface compared to the attached molecule, which is a specific binding partner for the particle of interest, see Figure 30. When a binding event occurs the refractive index changes leading to change of the angle of reflection of the light which can be detected. Both size and concentration can be determined from the detection (Wang *et al.* 2020).

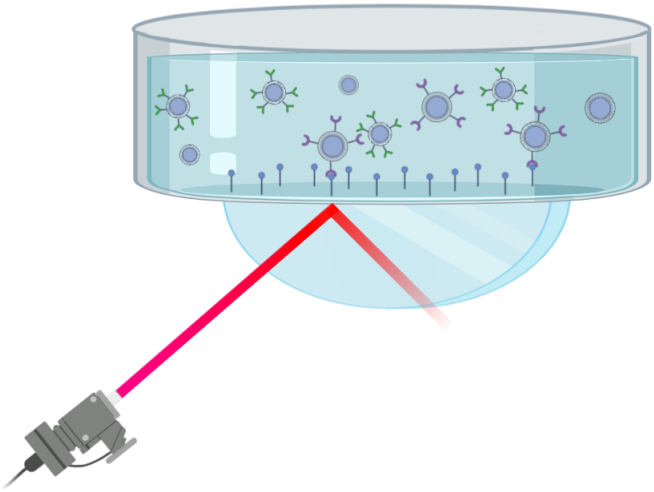

Figure 30. Schematic illustration of Surface Plasmon Resonance. A laser beam is focused on the opposed side of a surface where a binding molecule is attached. If a surface component of a vesicle binds the refractive index changes and the molecule can be detected.

8.3.3.1 Benefits can be identified but also challenges

There are a number of benefits using SPR for detection of exosomes, for example that it can measure total mass, is selective to different kinds of vesicles and can be used to analyze dilute samples (Rupert *et al.* 2014). Moreover it offers control of flow speed (Rupert *et al.* 2014) and is a label-free method that will not damage the molecules (Wang *et al.* 2020).

SPR is an accurate method and it has been proven that it has a precision of better than 5 % for protein concentration detection (Sjoelander & Urbaniczky 1991). Exosomes are harder to quantify than proteins partly because of deformation of the vesicles upon binding to the surface which interfere with the measurement (Rupert *et al.* 2014). Thus the concentration measurement is determined by Rupert *et al.* (2014) to have an accuracy be well within 50 % and it has a high signal-to-noise ratio. The disadvantages with this method are for example that the composition of lipids, proteins and nucleotides have a non-negligible impact on the binding as well as the

heterogeneity in size of exosomes (Rupert *et al.* 2014).

8.3.3.2 Different Surface Plasmon Resonance-based methods and platforms available

There are different SPR methods and platforms extending the use of SPR to detect exosomes. Examples of different assays are Nanoplasmonic Exosome (nPLEX) Assay (Pick *et al.* 2018) and Localized Surface Plasmon Resonance Imaging (LSPRi) platform (Wang *et al.* 2020). Both methods are real-time, high-sensitivity technologies that can be used to quantify exosomes and get a protein profile or digital responses. nPLEX can be used as an alternative to Enzyme-Linked ImmunoSorbent Assay (ELISA), which is the golden standard for protein measurements (Im *et al.* 2014). It requires less sample, is faster and more sensitive then ELISA. The LSPRi platform uses nano-sensors and is able to detect exosomes with higher accuracy then nPLEX (Raghu *et al.* 2018).

8.3.4 Accurate concentration measurements with Resistive Pulse Sensing

Resistive Pulse Sensing (RPS) is more accurate in concentration determining than for example NTA and Flow Cytometry (van der Pol *et al.* 2014) It measures transient change in current as individual particles pass through a pore in a membrane (Pick *et al.* 2018), see Figure 31. The method is based on the Coulter counter principal (Pick *et al.* 2018). There are different commercial instruments available, making it possible to both detect and characterize individual vesicles as well as bulk analysis (Pick *et al.* 2018). The obtained measurements can be used to calculate size of the particles, with a minimal detectable size of 70-100 nm. Size is determined by measuring how much the charge is changed when a particle passes the membrane and concentration is measured by counting the number of times the charge changes (Maas *et al.* 2014).

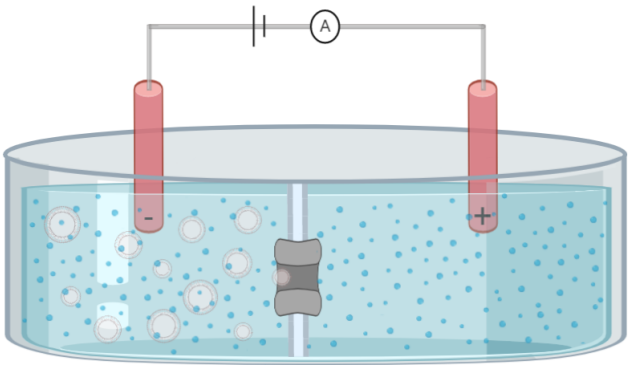

Figure 31. Schematic illustration of Resisitive Pulse Sensing. Vesicles, represented in white, travel through a pore, represented in black, in a membrane. The vesicles can be detected when the current changes. Positive and negative ions are present in the solution, represented by blue dots.

8.3.4.1 There are limitations to take into consideration

There are limitations for RPS, one example is that measurements can be affected by clogs and poor stability of the pores, which can make the measurement process take longer (van der Pol *et al.* 2014). The measurements are also affected by the large variety of sizes since vesicles of different sizes cannot be evaluated with the same pore

size (van der Pol *et al.* 2014). In This is one of the reasons why the tunable elastomeric pore sensing method is used.

8.3.4.2 Some limits can be overcome - then there are advantages

Tunable elastomeric pore sensing or Tunable Resistive Pulse Sensing (TRPS) method is based on the same concept as RPS with the difference that the pores are size-tuneable (Vogel *et al.* 2011, Maas *et al.* 2014). This feature of the pores makes TRPS suitable for analysis of exosomes, due to the heterogeneity of size between different EVs, since it makes it less likely that the pores become clogged. A drawback for this method is that it uses bulk analysis (Pick *et al.* 2018). The biggest advantages for the method is that you are able to use small samples, have relatively short measuring times and there is no need for purification before measuring (Maas *et al.* 2014).

8.3.5 Examination of molecular composition with Raman Spectroscopy

Characterization of molecular composition can be important because it can be used to identify EVs (Johnsen *et al.* 2014). Raman Spectroscopy is a method based on light capture of molecules in a solution (Wang *et al.* 2020), see Figure 32. It is a laser inelastic scattering technique that give an identifying fingerprint of the scattering patterns from the molecule. Raman Spectroscopy can be used to examine chemical components, such as lipids, cholesterol, carbohydrates and so on (Pick *et al.* 2018). It has been shown that lipid vesicles as small as 50 nm can be detected and that the measurements include quantitative measurements, size and shape (Bendix & Oddershede 2011). Since exosomes are in the range of 30-200 nm (van der Pol *et al.* 2014, Vlassov *et al.* 2012) a detection limit of 50 nm would detect most exosomes, which is an advantage for this method.

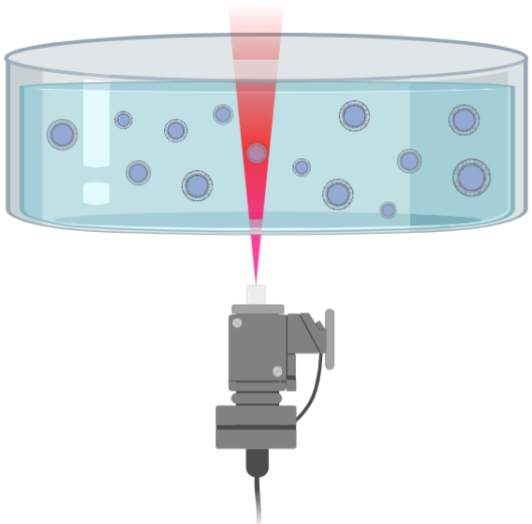

Figure 32. Schematic illustration of Raman Spectroscopy. A laser beam going through a sample with particles giving an identifying scattering pattern.

8.3.5.1 There are different methods with increased vesicle detection ability

Raman Spectroscopy is an inefficient method for detecting EVs because as little as 1 in 10^{6-8} photons are scattered (Stremersch *et al.* 2016). Due to the inefficiency, several different methods based on this technology have been developed that are more efficient. One of these methods is Surface Enhanced Raman Spectroscopy (SERS), which enhances the detectable signal by up to 10^{14-15} times (Stremersch *et al.* 2016). This method also have relatively short detection time but the EVs have to be coated with silver or gold (Wang *et al.* 2020). This method can be used for both bulk and single vesicle analysis.

Another method for single vesicle analysis is Laser Tweezers Raman Spectroscopy (LTRS). LTRS can use labels, but have as long detection time as 5 min for each particle (Smith *et al.* 2015). Yet another method described by Wang *et al.* (2020) is Single Particle Automated Raman Trapping Analysis (SPARTA). It is described as a new method that can give information about size as well as molecular composition.

8.4 Other methods widely used for detection and analysis of exosomes and lipid nanoparticles

There are a lot of methods available on the market with the potential of detection and characterization of exosomes and LNP. The methods mentioned above are only a small fraction of the methods used in research, clinical applications and industry. The methods have been chosen based on the potential they are described to have and the number of times they have been found in different articles. Potential for the methods have been evaluated on how developed and tested the methods are and if the method can be used not only in research to study interactions, composition or small samples. Other methods found in this project not considered interesting enough to be included in the report are presented short in Appendix C. Examples include:

- Digital methods including magnetic beads and/or different kinds of assays (Wang *et al.* 2020)
- Atomic Force Microscopy (Marquele-Oliveira *et al.* 2016)
- Electron microscopy (EM): Cryo-EM (Pick *et al.* 2018), Transmission EM (Vogel *et al.* 2011, Lv *et al.* 2018), Scanning EM (Vogel *et al.* 2011, Marquele-Oliveira *et al.* 2016)
- Mass spectroscopy (Abramowicz *et al.* 2016, Ha *et al.* 2016)
- Photon correlation spectroscopy (Marquele-Oliveira *et al.* 2016)

8.5 Which method to choose for detection and analysis of exosomes and lipid nanoparticles

Several different methods have been presented in this section and they have all different possibilities for detection and characterization of exosomes and LNP. They do also have different limitations and can determine diverse features of the vesicles. There is no one standardized method to date, which makes it challenging but also

opens for eligibility if limitations and possibilities are considered. Further you must decide what features to detect in which stage of production of exosomes or LNP.

8.5.1 One main problem is size limit

One of the main problems for the methods presented is that many of them have a size limit in the range of the size of exosomes and LNP. van der Pol *et al.* (2014) conclude that the accuracy of concentration measurements mainly depends on the minimal detectable size. Other problems are the heterogeneity in size and surface components and the small amount of surface proteins in exosomes. These factors have been identified as constraining and need to be overcome or made aware of. The different methods have different solutions to these problems but none of the methods are up til now developed enough to overcome them completely. Some of the methods can detect small particles, other are better at detection of single vesicles with high accuracy and some have solutions to reduce the impact of the small amount of surface components.

8.5.2 Bulk or single vesicle analysis?

Important parameters to consider is the type of measurement, if you should use bulk or single vesicle analysis. Based on the developed methods and the character of the samples obtained from production of both exosomes and LNP group we would recommend you to choose a method that uses single vesicle analysis. All mentioned methods, NTA, Flow Cytometry, SPR, RPS and Raman Spectroscopy have platforms or assays that are able to perform single vesicle analysis but this is not the case for all available platforms and assays on the market. It is important to be able to detect differences between different vesicles to be able to determine if purification steps where successful for exosomes and to see if the sample is monodisperse for LNP.

8.5.3 When to use labeling methods

When detecting exosomes there are methods that uses labeling and label-free methods. Labeling techniques can also be used for LNP if there are components that is possible to target. The advantage of using labeling methods is that you can be very specific in detection of particular exosome subpopulations. This is good when you want to know if the cells are producing the correct exosomes, and in what concentration. It can be good to use this kind of detection tool in early steps of production, and a way to determine when to harvest the cells. According to Patel *et al.* (2017) the concentration and other characteristics of the EVs are highly correlated with when the cells are harvested. Though it is not desirable to only detect one subpopulation after purification because it is important to be able to determine if there are any contaminants before using it as a drug. This means that a combination of detection tools could be beneficial.

8.5.4 The most developed methods are recommended

NTA and Flow Cytometry are the most developed and used methods for detection and analysis of exosomes and LNP which make them interesting as choice, but the other methods also have advantages and before ruling them

out detection parameters should be carefully considered. Both methods, NTA and Flow Cytometry, have the ability to analyze single vesicles and can use labels if wanted. They are commercialized and fast developing with small minimal detectable size, which is also important for correct concentration determination. The benefit of choosing NTA is that it is fast, and the benefit of choosing Flow Cytometry is the small detection limit.

It can be concluded that the field of analysis and characterization of exosomes and LNP is fast progressing and that new methods are developed at high rate, pushing the limits of size, concentration and required time lower and lower.

9 Conclusions

The goal of this project was to assemble a comprehensive analysis of exosomes and lipid nanoparticles as well as establish aspects that can be in favor for targeted drug delivery and the development of products at Cytiva. We can conclude that both exosomes and LNP show great potential as drug delivery vehicles. The advantage of using exosomes is that the vesicle naturally posses great biostability, low immunogenicity and toxicity in contrast to LNP. However, there are major challenges to large scale production of exosomes, such as the lack of efficient and optimized production- and purification strategies. Thus, the development of new and advanced standardized methods is required to meet the growing demand on the pharmaceutical market. The knowledge about structure and targeting ligands of the vesicles can facilitate the entry into the manufacturing field of targeted drug delivery.

9.1 Exosomes as drug delivery vehicles

In addition to the great biostability and low immunogenicity, exosomes also possess the ability to cross biological barriers as well as to encapsulate therapeutic compounds. These characteristics and the structural composition makes the exosome a suitable option as a drug delivery vehicle. However, to make use of exosomes as drug delivery vehicles, efficient methods for cargo loading as well as modifications to assure specific targeted delivery is required. There are several suggested methods for cargo loading, depending on what cargo is loaded. Generally, it is easier to load small molecules and more difficult to achieve successful loading of larger, more complex compounds. A more efficient targeted delivery can be achieved using different modification strategies of exosome surface components, for instance enrichment of targeting ligands by induction of an overexpression, or fusion of targeting ligand to naturally occuring transmembrane molecules.

9.1.1 Exosome production

A big challenge for exosomes to reach the clinic is to produce sufficiently large amount of exosomes with constant characteristics. As of today, there is no large-scale production of exosomes but the cell culture method

that shows most potential is perfusion-based Hollow-Fiber Bioreactor.

9.1.2 Exosome purification

The purification step in exosome production is essential to avoid contaminants that highly affects the pharmaceutical release profile and aggravate correct dosing. There is no purification technique today that is "perfect" to purify exosomes. Some important features that must be taken into consideration in clinical applications is low contamination, isolation of specific subpopulations and scalability. SEC alone is the most suitable technique today due to its scaling possibilities, easily adaptable equipment and low breakage of exosomes.

9.1.3 Exosome analysis

To assure adequate quality of the drug delivery vehicles correct analysis and detection of vesicles is crucial. It is important to choose the correct detection methods both after production to mesure concentration of exosomes and after purification for purity determination. When detecting exosomes important characteristics are size, concentration and surface components. NTA and Flow Cytometry are the most developed and used methods for detection and can analyze single vesicles and use labels if wanted.

9.2 Lipid nanoparticles as drug delivery vehicles

Lipid based nanoparticles have also proven to be successful drug delivery vehicles. The vesicular structure is easy to moderate using both surface-attached biological ligands and incorporated transmembrane additives. Lipid based nanoparticles generate few side effects and exhibit low toxicity when distributed *in vivo*. To further increase site-specific LNP accumulation during treatments, a variety of disease-specific features are identified and the matching counterpart is added to the LNP surface. LNP provide opportunity to deliver high concentrations of lipophilic and hydrophilic drugs and have demonstrated the ability to cross membranes and impenetrable barriers which makes them desired on the market of novel drug delivery systems.

9.2.1 Lipid nanoparticle production

The selection of production method for LNP is highly affected by the desired characteristics and the required loading efficiency. Microemulsion and High-Pressure Homogenization are the most well-established methods and have multiple advantages including scalability and availability of instruments. However, if monodispersity for release profile control is required, methods using organic solvents e.g. Supercritical Fluid Extraction are preferred. LNP have the advantages of not requiring extensive purification.

9.2.2 Lipid nanoparticle analysis

Detection and analysis of LNP is crucial to assure adequate quality of the drug delivery vehicles. When detecting LNP important characteristics are size, concentration and size dispersity. The most developed and used methods are NTA and Flow Cytometry which can perform single vesicle analysis.

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11 Contribution statement

All project members contributed to the project plan. S Lundberg and E Karlsson presented the project plan.

The literature study was conducted by all members, and all aspects of the project have been discussed with the entire group. All members have also contributed with feedback to one another. The report was written by all project members where:

- K Carlsson wrote about exosome biogenesis and exosomes as drug delivery vehicles, focusing on cargo loading.
- Sa Larsson wrote about exosome structure and exosomes as drug delivery vehicles, focusing on specific delivery.
- M Glansk focused on lipid nanoparticle structure and important modifications, important for drug carriers and for specific targeting.
- S Lundberg contributed by describing methods and strategies for production of lipid nanoparticles.

- H Dahlberg focused on methods and strategies for exosome production.
- E Karlsson wrote about purification strategies for exosome production.
- So Larsson contributed by describing methods for analysis and detection of exosomes and lipid nanoparticles.

The half-time report was compiled and finalized by M Glansk and So Larsson after contribution from all project members. The ethical analysis was written by S Lundberg. The poster was made and presented by K Carlsson. The final project presentation was made by M Glansk, Sa Larsson, So Larsson and H Dahlberg.

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A Ethical analysis

A.1 Does single-use technologies equal sustainability?

The generated waste per unit of product in pharmaceutical production has one of the highest value of all chemical industries (Rajagopal & Rajagopal 2014). Not only does this affect the financial situation of the pharmaceutical companies but also, the task of waste disposal is extensive (Ganesan & Narayanasamy 2017). Consequently, there are numerous environmental aspects that have to be taken into consideration in the development process of imminent drugs.

At first thought, single-use technologies might not rhyme with environmentally friendly due to issues concerning disposal of products. However, in pharmaceutical manufacturing, there are several benefits of single use products. These factors have in some cases shown to have less impact on the environment despite the complications of destruction of material. The advantages of single-use products may include; reduction in energy input and water use in the manufacturing stage. Are single-use products always ethically preferable or should stainless steel equipment be used in most situations? The life cycle assessment (LCA) study from Cytiva in 2012 has one significant conclusion, "single-use and sustainability go hand in hand" (Cytiva 2012). Climate change, energy inputs, freshwater consumption, human health, natural resources and the quality of ecosystems were factors taken into consideration when conducting the study (Cytiva 2012).

The result of the study showed that single-use technologies *usually* resulted in less impact on the environment compared to stainless steel technologies. Why is this not always the case? The environmental impact of single-use products is highly affected by the availability of locally produced products. If the single-used products have to be transported a great distance, the use of them is discouraged due to carbon emissions. Also, what type of vehicles are used for the transport? Airplanes pitch in with an intensive amount of carbon emissions and many companies use them as primary transportation vehicles. Transport is not the only contributor to environmental damage, the choice of material for the single-use products affects the extent of the impact.

A.2 Development of new materials crucial for increased sustainability

Is it possible to develop more environmentally friendly single-use products to make the process even more sustainable? The best option would be to develop effective biodegradable single-use products that do not affect the environment upon disposal. However, that is probably something that will take a very long time to develop and at the moment there are no such reasonable low-cost products. There are still some materials that should be completely avoided that are really harmful for the environment and in many cases there are probably products from some steps of the production line that can be changed to more environmental friendly materials despite a slight increase of cost.

A.3 High energy approaches can increase the carbon footprint

Whether to use single-use or stainless steel is not the only question relating to environmental aspects in the production of exosomes and lipid nanoparticles. Some of the methods used for the production of exosomes and LNP are energy intensive. There are numerous techniques and they all require different energy inputs and solvents. What production method leads to the smallest carbon footprint? To keep the facilities and instrument running both electricity and fuel are required. The impact of this is highly affected by the source of energy. How can we choose or develop the existing methods so that the production line will still be effective but also sustainable and thereby ethically justifiable? By using alternative energy sources, the environmental footprint can be reduced (Ganesan & Narayanasamy 2017). If two methods generate equally effective particles and the more sustainable one of the two cost more, how should companies evaluate that information? If that's the case, perhaps a compromise is the best option. The companies could then use a combination of two different energy sources. Another question is, if the methods themselves are highly energy intensive, does the energy requirement of the production of single-use products matter? Due to the current environmental situation of the earth, most people would agree that every contribution, no matter how small, makes a difference.

A.4 Water from facilities will end up in all water sources

The water used for cleaning of instruments in stainless steel industries will eventually end up in nature. The residual pharmaceuticals that enter the seas and other water sources will affect the aquatic life and subsequently humans that e.g. use fish as food sources.

World Health Organization (2017) has estimated that by 2025, 50 % of all the humans on earth will live in water-stressed regions. Therefore, it is of great importance to decrease the water consumption in the manufacturing industry and facilitate for the regions where drinking water must be the highest priority. The LCA-study showed that the impact of the water supply for the manufacturing industries was highly affected by the geographical location (Cytiva 2012).

The production of solid lipid nanoparticles (SLN) requires a relatively low amount of water used for solvents. An example of an U.S Federal Drug and Food Administration (FDA) approved drug that has been encapsulated in SLN is Darunavir, an antiretroviral drug against HIV. The daily dosage of Darunavir is 800 mg, according to FASS (FASS 2019). The aqueous phase in the production of 80 mg Darunavir is approximately 20 ml (Desai & Thakkar 2016). The 90 % encapsulation efficiently leads to a yield of 72 mg. This means that (800/72)*20 = 222 ml water is needed for one person's daily consumption of Darunavir. To put these number in perspective, 1700 litres of water are used to produce a 100 g chocolate bar (National Geographic 2015). This means that the amount of water used in the production of one chocolate bar can be used to treat 1700/0,222 = 7658 people per day.

These calculations do not take cleaning of instruments into consideration and strictly give an overview of the

amount of water used as material in the production stage. Further, the encapsulation efficiency and yield are not the same for every drug encapsulated in lipid nanoparticles and exosomes. The calculations are simply used for a comparison and not exact numbers. As there are no FDA-approved drugs past clinical trials that are encapsulated in exosomes the same calculations is not possible for exosomes. However, the amount of water will be significantly higher due to the large volumes of cell cultures in the manufacturing process of exosomes.

A.5 Organic solvents leave permanent marks in the environment

In some of the methods for lipid nanoparticle production, the use of organic solvents is required (Naturvårdsverket 2019). Organic solvents that are released in the environment damage the surrounding environment which subsequently affects the nature, animals and humans (Naturvårdsverket 2019). Areas at a great distance from the emission source are affected by the cursory environmental toxins that can travel across the globe. Organic solvents do not disappear with time and invariably alter the surrounding environment (Naturvårdsverket 2019). An observed issue the last few decades are organic solvent residues from the pharmaceutical manufacturers that have been aggregated in different watercourses, including the Baltic Sea. The use of organic solvents in Sweden are regulated and the ordinance includes limitation values of releases from different type of businesses. The question is, does single-use products prevent the release of organic solvents into the environment? When the stainless steel equipment is cleaned there is a risk of residual organic solvents being transported to the surroundings along with the water. However, the way of disposal of the single-use products also affects the spreading of the organic solvents.

A.6 Single-use or stainless steel - does it have to be one or the other?

Every pharmaceutical company wants to have an efficient and fast production. The question is, could a combination of both approaches be the best option? The LCA study performed by Cytiva in 2017 (Cytiva 2012) showed that single-use products had less impact in the use-stage but an increase of impacts in the higher supply chain.

To see the bigger picture in exosome and lipid nanoparticle production, research of all the steps of the production line, including purification and analysis are equally important. One technique might be more suitable for single-use products than the other. One option is to substitute some processes in the manufacturing step and preserve the stainless steel approach for others. In this way, there's a possibility to streamline the process and make it more cost effective. In addition, it can be a good start for companies that want to optimize the manufacturing and still ensure product quality.

A.7 Do the benefits of disease treatment contradict a sustainable production?

The question about the environment is currently discussed on all levels in the world, from dining tables to the leaders of the United Nations. The ethical issue in this debate is whether the response of future generations lay upon the people living today and how to consider the balance between justice of the people living today and future generations. Many people agree on that it is important to take consideration in actions and choices made today to preserve nature for future generations.

The importance of preserving nature for future generations has to be balanced with the determination of curing diseases. Exosomes and LNP are used as vehicles in drug delivery. Many of these drugs can be crucial for the survival of severely sick people. If the exosomes and LNP are used to cure diseases, how can that be evaluated in contrast to the environmental damage? What is the most important for society? And are all drugs considered to be equally important? In theory, the companies that produce drug delivery vehicles might not always be responsible for the packing of drugs. Therefore, it will be difficult to use the argument of crucial drugs since the vehicles can be packed with drugs that are considered "less" important for some people. Should a company that packs the vehicles have more strict regulations of water use and disposal of materials? And is it their responsibility only?

A.8 Ethical environmental aspects for biotechnology companies

In order to keep in the frontline of list of environmental companies, it's important to stay updated on the latest techniques and products. To take the whole value chain of the products into consideration is a positive strategy that will positively influence the green pharmaceutical market. The LCA-study from Cytiva (2012) included two technologies and if Cytiva wants to be influential in the green industry, our proposal is to continuously update the studies and broaden the research to more fields/techniques. In conclusion, embrace new methods and products and don't be too conservative. If not, there's a risk of money loss, contribution to excess water use or causing significant harm on the environment.

B Production methods of lipid nanoparticles

Table B.1. Overview of the advantages and disadvantages of different production methods of lipid nanoparticles (LNP), Solvent Diffusion, Solvent Injection, Coacervation and Phase Inversion Temperature (PIT).

Production method	Mechanism	Advantage	Disadvantage	Reference
Solvent Diffusion	Rapid diffusion cause LNP formation.	• No need of extensive instruments • Small LNP sizes • Low polydispersity • Simple to scale-up • Avoidance of heat	• Risk of toxicological issues due to organic solvents • Low content of lipid • Low degree of dispersion • Instability issue of emulsion • Solvent removal step	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015)
Solvent Injection	Rapid diffusion by injection cause LNP formation.	• No need of extensive instruments • Effective and versatile process • Simple handling and fast process • Simple to scale-up • Avoidance of heat • Low energy input	• Risk of toxicological issues due to organic solvents • Low content of lipid • Solvent removal step	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015, Yoon *et al.* 2013)
Coacervation	Acidification cause a decrease in pH in a micellar solution. The proton exchange leads to lipid nanoparticle precipitation.	• Convenient for lipophilic drugs • No need for solvents • Monodispersity • Simple to scale-up	• Not applicable for pH-sensitive drugs	(Shah *et al.* 2015)
Phase Inversion Temperature	Thermal variation treatment by heating and cooling cycles promotes spontaneous inversions of emulsions which leads to lipid nanoparticle formation.	• No need of large volumes of surfactants • No need for solvents • Suitable for thermo-labile drugs • Low energy input	• Risk of LNP aggregation • Instability of emulsion • Addtional molecules can easily affect the inversion	(Ganesan & Narayanasamy 2017, Shah *et al.* 2015, Yoon *et al.* 2013)

C Analysis methods

Table C.1. A compilation of methods found that can be used for detection of exosomes and lipid nanoparticles not included in the report. The list is not exhaustive, more methods could be found.

Technique	Description	Exosome (E) or Lipid Nanoparticle (LNP)	Advantage	Disadvantage	Reference
Microscopy methods					
Digital methods	Methods where a microscopic digital detection technique is used to detect molecules that have hybridized to the surface of the exosome. The molecules can for example be magnetic beads or DNA. A microwell assay can be used to ensure detection of singular vesicles.	E	Specific and accurate method.	Only tested on specific diagnostic application.	(Wang *et al.* 2020)
Atomic force microscopy	Characterize the surface of the particles and the size. Mechanical probe feels the surface and can thereby detect the particles.	E, LNP	Gives a very precise picture of the sample, interactions, surfaces and so on.	Harder to use in larger samples. Give a very accurate but small picture of the sample.	(Marquele-Oliveira *et al.* 2016)
Cryo-EM	Structure and size determining of vesicles. Sample frozen before measurement.	E, LNP	Gives a detailed picture of the vesicles without disrupting the membrane.	Hard to analyze over large sections.	(Pick *et al.* 2018)
Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM)	Structure and size determining by scanning or transmission.	E, LNP	Gives a detailed picture. Used a lot in research.	Hard to use in scale up experiments, more for analysis in studies. Complement that can confirm other results.	(Vogel *et al.* 2011, Marquele-Oliveira *et al.* 2016, Lv *et al.* 2018)
Spectrometry methods					
Mass Spectrometry (MS)	Fragments molecules and separates on charge and size in an electromagnetic field.	E, LNP	Standard method. Get a precise picture of the content that can be used to analyze both exosomes and their content.	Even small contaminants affect the analysis.	(Abramowicz *et al.* 2016, Ha *et al.* 2016)
Optical methods					
Fluorescence Correlation Spectroscopy (FCS)	Characterizing of molecular interactions.	E	Highly used method.	Can be hard to fit a curve because EVs are heterogeneous. A method manly to study function of exosomes.	(Pick *et al.* 2018)
Photon Correlation Spectroscopy (PCS), Dynamic Light Scattering	Measure light scattering. Dynamic measurement. Can detect fluorescing molecules on the surface of the exosome. Both for size and shape determining.	E	Can be used to confirm results from e.g. NTA.	Sensitive to contaminants, they can easily interfere with the measurement. Often rejected in favor of other more developed light scattering methods.	(Buzás *et al.* 2017)

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Technique	Description	Exosome (E) or Lipid Nanoparticle (LNP)	Advantage	Disadvantage	Reference
Frequency Locking Optical Whispering Evanescent Resonator (FLOWER)	A microtoroid coupled to an optical fiber. When nanoparticles bind to the toroid the interaction between the light in the toroid and the fibre changes which leads to detection.	E	Able to detect with a very small margin of error.	Have only been described used in diagnostic applications.	(Pick *et al.* 2018)
Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS)	Detect surface bio-markers and size. Single vesicle analysis method. Can use different antibodies to detect different vesicles. Gives information about both size and phenotype.	E, LNP	Label-free, can detect different characteristics from the same assay.	Need antibodies for all components that are wanted to detect. Not mentioned in lots of articles.	(Pick *et al.* 2018)
Ellipsometry	Optical method that examine dielectric properties (refractive index, dielectric function) on thin film. Measure change in polarization of light.	E, LNP	Cheap and easy to perform.	Not scalable.	(Rupert *et al.* 2014)
Optical Waveguide Light-Mode Spectroscopy	Enables *in situ* measurements of immobilized bio-molecules in solution. Spectroscopy method.	E, LNP	Cheap and simple.	Not as accurate. Only get an average over the whole sample.	(Rupert *et al.* 2014)
FT-IR	A spectroscopy method that measures the absorbed energy from infrared light.	E, LNP	Gives a picture of chemical composition.	Samples milled and mixed.	(Marquele-Oliveira *et al.* 2016)
Chromatographic methods					
Densometry Analysis	A combination of TLC and colon-chromatography for relative quantitative measurement.	E, LNP	Relatively cheap. Both quantitative and qualitative.	Not for scale up experiments.	(Buzás *et al.* 2017, Sawada *et al.* 2020)
High Pressure Liquid Chromatography (HPLC)	A chromatography method that separates a sample based on different properties.	E, LNP	Can be used to separate components on hydrophobic/hydrophilic properties.	Do not give information specific to exosomes, could include other types of vesicles.	(Marquele-Oliveira *et al.* 2016)
Thin Layer Chromatography (TLC)	Measure purity of lipids.	E, LNP	Purity analysis, cheap and fast.	Not quantitative, not for scale up experiments.	Sawada *et al.* (2020)
Protein and lipid measurements					
Colormetric Protein Assay	Measure protein concentration in solution.	E, LNP	Can detect in UV range. Simple, cheap and fast method.	Not for scale up experiments. Can also include nucleic acids.	(Rupert *et al.* 2014)
Western Blot	Method to separate proteins and then transfer them to a membrane. Add a probe with a marker to the membrane that attaches to the protein.	E	Can confirm results from e.g. EM by looking at specific surface proteins in exosomes.	Only give information about that one protein is present in the solution.	(Lv *et al.* 2018)

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Technique	Description	Exosome (E) or Lipid Nanoparticle (LNP)	Advantage	Disadvantage	Reference
Choline Oxidase	Measure amount of lipids in solution.	E, LNP	-	Not enough to determine amount of lipids in solution.	(Sawada *et al.* 2020)
Other measurements					
Differential Scanning Calorimetry (DSC)	Measure how much energy it takes to increase the temperature.	E, LNP	Gives information about interactions.	Measurements not that important for analysis in production.	(Marquele-Oliveira *et al.* 2016)